US006316274B1

(12) **United States Patent**
Herron et al.

(10) **Patent No.: US 6,316,274 B1**
(45) **Date of Patent: Nov. 13, 2001**

(54) **APPARATUS AND METHODS FOR MULTI-ANALYTE HOMOGENEOUS FLUORO-IMMUNOASSAYS**

(75) Inventors: **James N. Herron**; **Douglas A. Christensen**; **Hsu-Kun Wang**; **Karin Caldwell**, all of Salt Lake City, UT (US); **Vera Janatová**, Prague (CZ); **Shao-Chie Huang**, Salt Lake City, UT (US)

(73) Assignee: **University of Utah Research Foundation**, Salt Lake City, UT (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/516,307**

(22) Filed: **Mar. 1, 2000**

Related U.S. Application Data

(62) Division of application No. 08/979,582, filed on Nov. 26, 1997, now abandoned, which is a continuation of application No. 08/748,687, filed on Nov. 13, 1996, now Pat. No. 5,919,712, which is a division of application No. 08/263,522, filed on Jun. 22, 1994, now Pat. No. 5,677,196, which is a continuation-in-part of application No. 08/110,169, filed on Aug. 20, 1993, now Pat. No. 5,516,703, and a continuation-in-part of application No. 08/071,579, filed on Jun. 2, 1993, now abandoned, which is a continuation-in-part of application No. 08/064,608, filed on May 18, 1993, now Pat. No. 5,512,492.

(51) **Int. Cl.**[7] **G01N 33/543**

(52) **U.S. Cl.** **436/518**; 422/55; 422/57; 422/58; 422/82.05; 422/82.08; 422/82.11; 435/287.1; 435/287.2; 435/287.9; 435/288.5; 435/288.7; 435/808; 436/164; 436/165; 436/527; 436/531; 436/805; 436/807

(58) **Field of Search** 422/55, 57, 58, 422/82.05, 82.08, 82.11; 435/287.1, 287.2, 287.9, 288.5, 288.7, 808; 436/164, 165, 518, 527, 531, 805, 807; 204/400, 403

(56) **References Cited**

U.S. PATENT DOCUMENTS

Re. 33,064	9/1989	Carter et al. .
Re. 34,394	9/1993	Bunting .
D. 426,783	6/2000	Christensen et al. .
3,939,350	2/1976	Kronick et al. .
3,959,078	5/1976	Guire .
4,050,895	9/1977	Hardy et al. .

(List continued on next page.)

FOREIGN PATENT DOCUMENTS

0 478 137	1/1992	(EP) .
0 517 516 A1	12/1992	(EP) .
0 519 623 A2	12/1992	(EP) .

(List continued on next page.)

OTHER PUBLICATIONS

#Carlsson, "Protein Thiolation and Reversible Portein–Protein Conjugation", *Biochem J.*, 173, pp. 723–737, 1978.

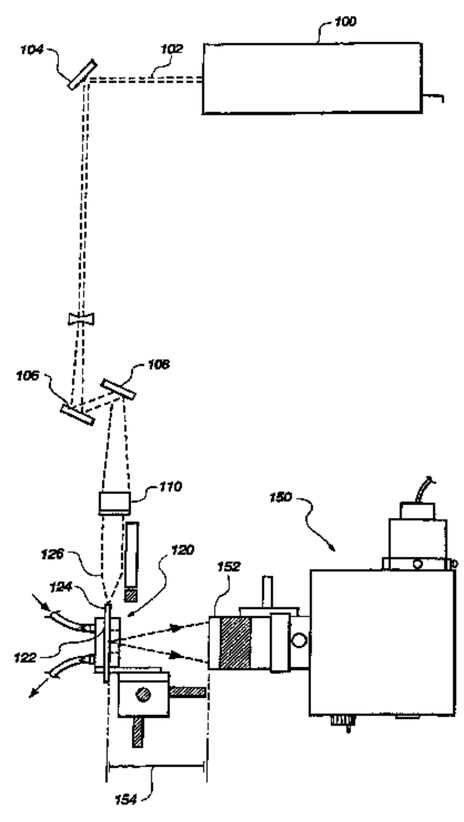
(List continued on next page.)

Primary Examiner—Christopher L. Chin
(74) *Attorney, Agent, or Firm*—TraskBritt

(57) **ABSTRACT**

Methods and apparatus for evanescent light fluoroimmunoassays are disclosed. The apparatus employs a planar waveguide with an integral semi-cylindrical lens, and has multi-analyte features and calibration features, along with improved evanescent field intensity. A preferred embodiment of the biosensor and assay method have patches of capture molecules each specific for a different analyte disposed adjacent within a single reservoir. The capture molecules are immobilized to the patches on the waveguide surface by site-specific coupling of thiol groups on the capture molecules to photo-affinity crosslinkers which in turn are coupled to the waveguide surface or to a non-specific-binding-resistant coating on the surface. The patches of different antibodies are produced by selectively irradiating a portion of the waveguide surface during the process of coupling the photo-affinity crosslinkers the selective irradiation involving a mask, a laser light source, or the like.

8 Claims, 14 Drawing Sheets



U.S. PATENT DOCUMENTS

4,166,105	8/1979	Hirshfeld .	
4,235,869	11/1980	Schwarzberg .	
4,264,766	4/1981	Fischer .	
4,298,685	11/1981	Parikh et al. .	
4,429,008	1/1984	Martin et al. .	
4,447,546	5/1984	Hirschfeld .	
4,450,231	5/1984	Ozkan .	
4,558,014	12/1985	Hirschfeld et al. .	
4,562,157	12/1985	Lowe et al. .	
4,596,723	6/1986	Kaufman et al. .	
4,627,445	12/1986	Garcia et al. .	
4,708,871	11/1987	Geysen .	
4,722,906	2/1988	Guire .	
4,775,637	10/1988	Sutherland et al. .	
4,806,312	2/1989	Greenquist .	
4,810,658	3/1989	Shanks et al. .	
4,820,649	4/1989	Kawaguchi et al. .	
4,826,759	5/1989	Guire et al. .	
4,852,967	8/1989	Cook et al. .	
4,880,752	11/1989	Keck et al. .	
4,909,990	3/1990	Block et al. .	
4,973,493	11/1990	Guire .	
4,978,503	12/1990	Shanks et al. .	
4,979,959	12/1990	Guire .	
4,997,278	3/1991	Finlan et al. .	
5,002,582	3/1991	Guire et al. .	
5,006,333	4/1991	Seifer et al. .	
5,006,716	4/1991	Hall .	
5,043,278	8/1991	Nagaoka et al. .	
5,061,857	10/1991	Thompson et al. .	
5,071,216	12/1991	Sullivan .	
5,071,217	12/1991	Birch .	
5,073,484	12/1991	Swanson et al. .	
5,081,012	1/1992	Flanagan et al. .	
5,096,807	3/1992	Leaback .	
5,135,876	8/1992	Andrade et al. .	
5,156,976	10/1992	Slovacek et al. .	
5,166,515	11/1992	Attridge .	
5,168,537	12/1992	Rajasekharan et al. .	
5,182,216	1/1993	Clayton et al. .	
5,186,897	2/1993	Eason et al. .	
5,192,502	3/1993	Attridge et al. .	
5,217,492	6/1993	Guire et al. .	
5,242,838	9/1993	Bergstrom et al. .	
5,248,620	9/1993	Sluka et al. .	
5,258,041	11/1993	Guire et al. .	
5,263,992	11/1993	Guire .	
5,344,784	9/1994	Attridge .	
5,380,668	1/1995	Herron et al. .	
5,436,161	7/1995	Bergstrom et al. .	
5,512,492	4/1996	Herron et al. .	
5,552,272	9/1996	Bogart .	
5,580,794	* 12/1996	Allen	436/169
5,677,196	10/1997	Herron et al. .	
5,814,565	9/1998	Reichert et al. .	
5,832,165	11/1998	Reichert et al. .	
5,846,842	12/1998	Herron et al. .	
5,919,712	7/1999	Herron et al. .	
5,961,924	10/1999	Reichert et al. .	
6,108,463	8/2000	Herron et al. .	

FOREIGN PATENT DOCUMENTS

2220083	12/1989	(GB) .
943673	10/1995	(M) .
WO 81/00912	4/1981	(WO) .
WO 89/09408	10/1989	(WO) .
9001166	2/1990	(WO) .
9006503	6/1990	(WO) .
9 113 339	9/1991	(WO) .
9115751	10/1991	(WO) .
9209892	6/1992	(WO) .
9 216 838	10/1992	(WO) .
WO 92/16838	10/1992	(WO) .

OTHER PUBLICATIONS

#Ciana, Leopoldo Della, "Electrochemiluminescence Immunosensors And DNA Probes For Clinical Diagnostics", Abstract, p. 19. No date.

Fitch, R., "Polymer Colloids: A Comprehensive Introduction", *Academic Press, Inc.*, 10 pages, 1997.

#Harmer, "Guided–Wave Chemical Science", *Electrochem. Soc.* 87–9 (Proc. Synp. Chem. Sens.), pp. 409–427, 1987.

#Harrick et al., "Multiple Internal Reflection Fluorescence Spectrometry", *Analytical Chemistry*, vol. 45, No. 4, pp. 687–691, Apr. 1973.

#Herron, "Fluorescent immunosensors using planar waveguides", *SPIE*, vol. 1885, Proceedings of Advances in Fluoresence Sensing Technology, pp. 28–39, 1993.

#Huang, Shao–Chie, "PEG derivatives as tethers for site–directed immobilization of oxidized antibody", title page, abstract (pp. iv–v), and pp. 35–39. No date.

#Huang, Shao–Chie, "PEG Derivatives As Tethers For Site–Directed Immobilization Of Oxidized Antibody", Master's Thesis, Nov. 1991.

#Ives, Jeffrey T., "Optical waveguide sensors: characterization of evanescent and scatter excitation," Ph.D. dissertation submitted to Dept. of Bioengineering, University of Utah, Jun. 1990.

#*J. Chromatography 604*: 63–71, Caldwell, Li, Li and Dalgleish, "Adsorption behavior of milk proteins of polystrene Itex", Elsevier Science Publishers, 1992.

#Lee, "Protein–resistant surfaces prepared by PEO–containing block copolymer surfactants", *Journal of Biochemical Materials Research*, pp. 351–368, 1989.

#Lakowicz, Joseph R., "Fluorescence Lifetime–Based Sensing And Imaging", Abstract, p. 5. No date.

#Leiner, M.J.P., "Optical Sensors For In Vitro Blood Gas Analysis", Abstract. No date.

#Ligler et al., "Evanescent Wave Optical Fibre Sensors", Abstract, p. 75. No date.

#Liu, Benjamin L., "Clinical Requirements For Optical Immunosensors", Abstract, p. 13. No date.

#Masuda, Kenji, "Study Of The Sensitivity Of Evanescent Wave Immunoassay", Abstract, p. 11.

#*Multiple Internal Reflection Fluorscence Spectrometry*, Harrick, N.J., and Loeb, George I., Analytical Chemistry, vol. 45, No. 4, Apr. 1973, pp. 687–691.

#O'Keeffe et al., "Development Of A LED Based Phase Fluorimetric Oxygen Sensor Using Evanescent Wave Excitation Of A Sol–Gel Immobilized Dye", Abstract, p. 149. No date.

#O'Neill et al., "Use Of A Optical Biosensor To Measure Prostate Specific Antigen In Whole Blood", Abstract, p. 20. No date.

#*Particle Size Distribution II*, Li, J., Caldwell, K.D., and Tan, J.S., Chapter 16, "Size analysis of a block copolymer-coated polystyrene latex", pub. American Chemical Society etym 1991.

#Robinson, Grenville, "The Commercial Development Of Planar Optical Biosensors", Abstract. No date.

#Sacco et al., "Fibre–Fluorescence Immunosensors Based On Evanescent Wave Detection", Abstract, p. 52. No date.

#Slovacek et al., "Application Of A Plastic Evanescent Wave Sensor To Immunological Measurements Of CKMB", Abstract, p. 16. No date.

#van Erp et al., "Affinity of monoclonal antibodies", *J. Immunol. Methods*, 140:235–241, 1991.

* cited by examiner

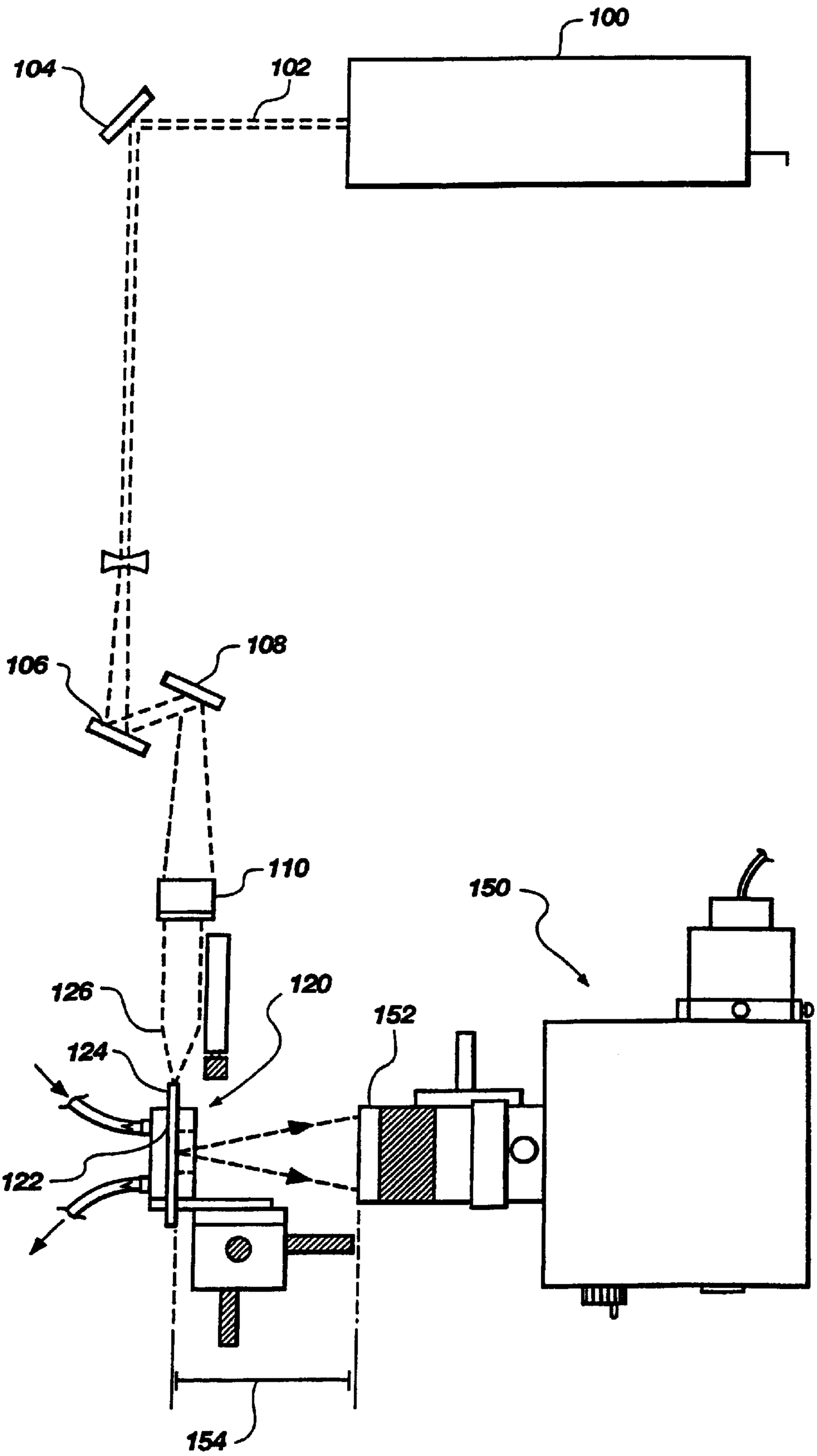


Fig. 1

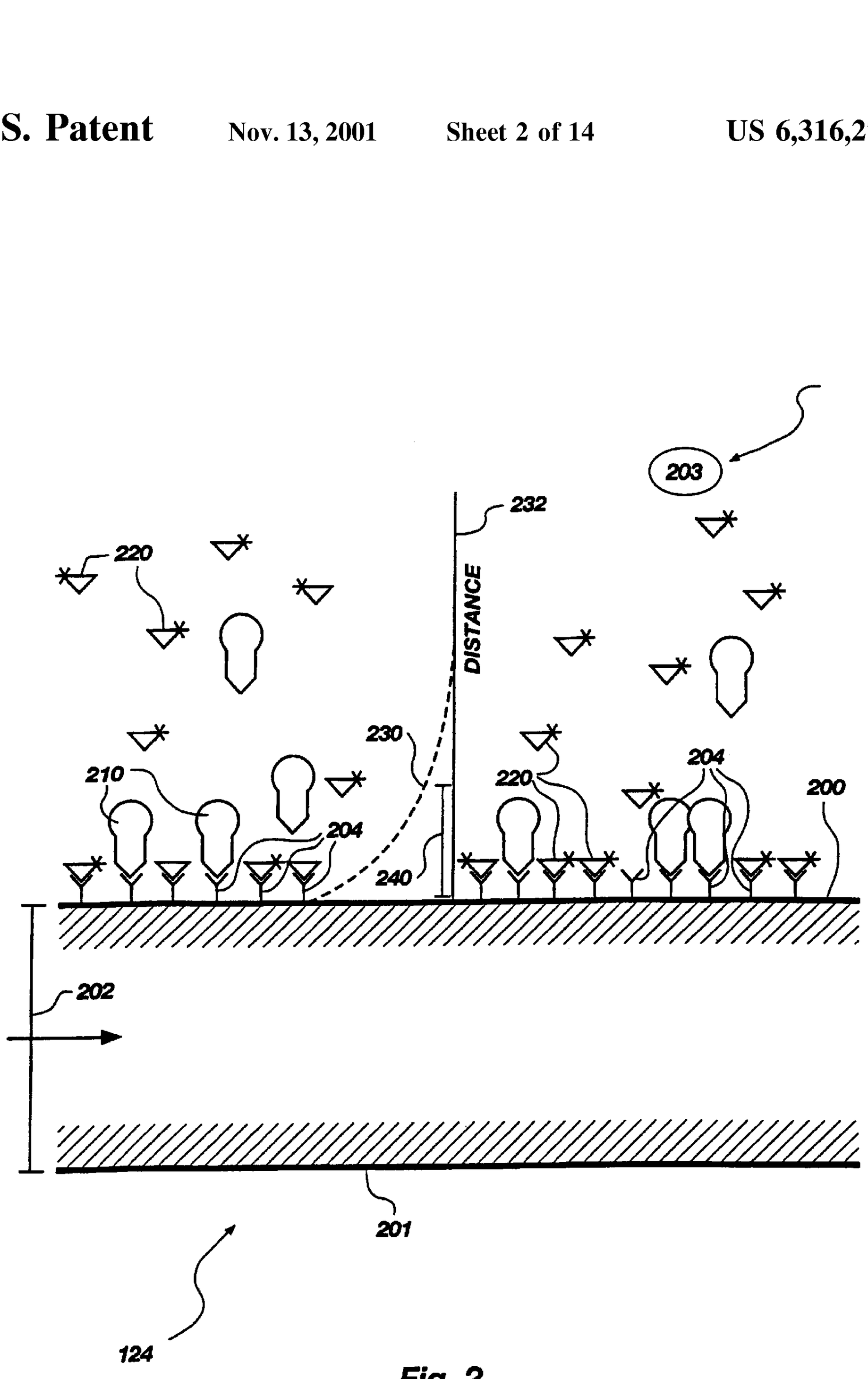


Fig. 2

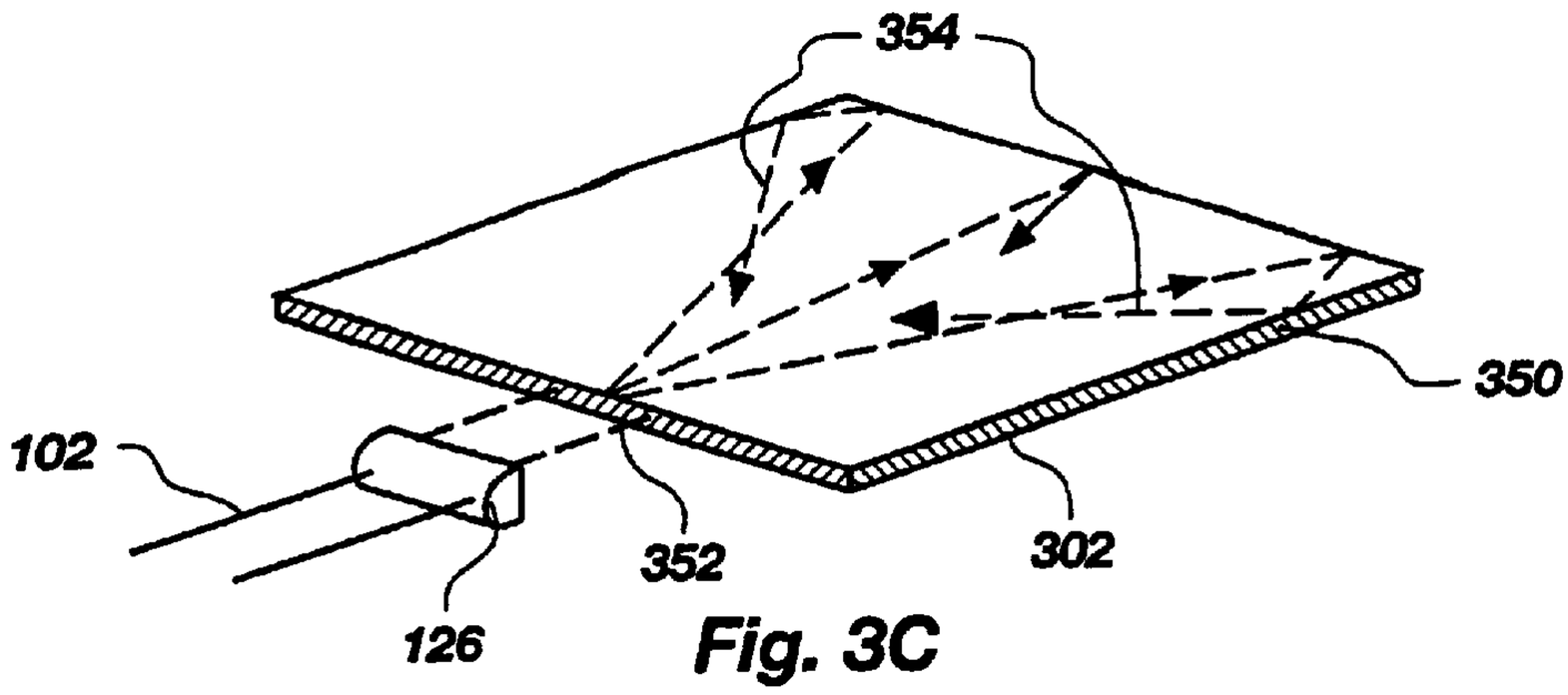


Fig. 3C

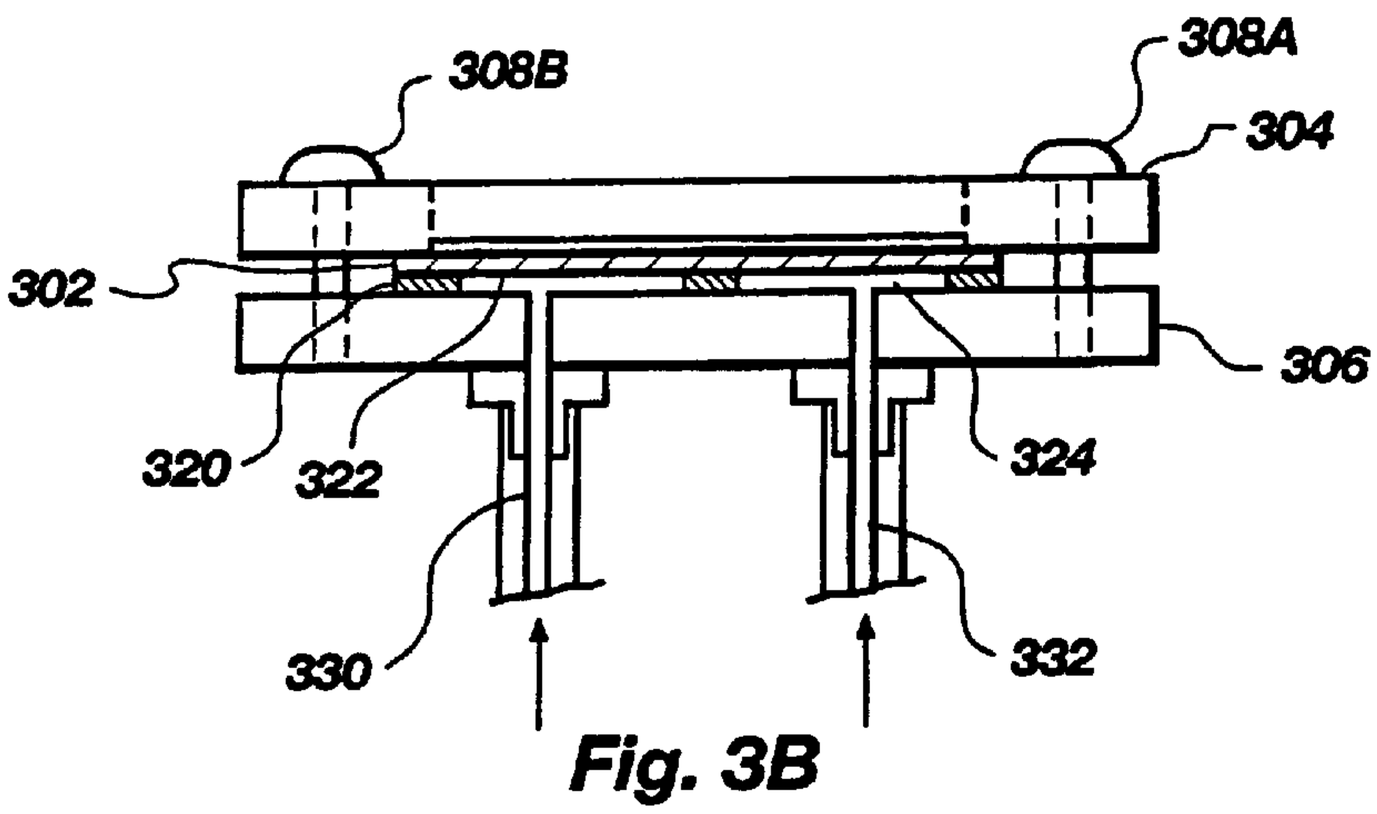


Fig. 3B

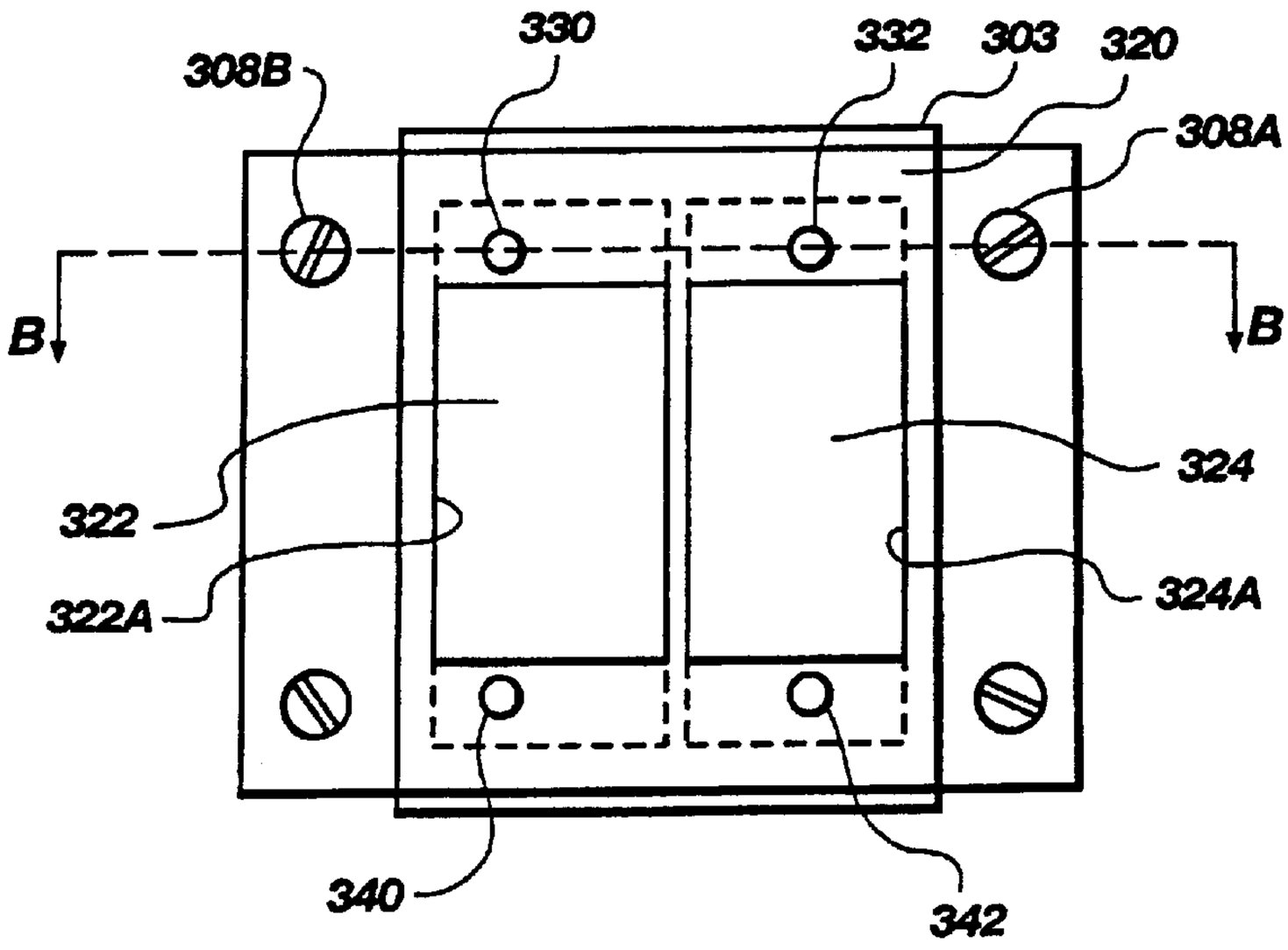


Fig. 3A

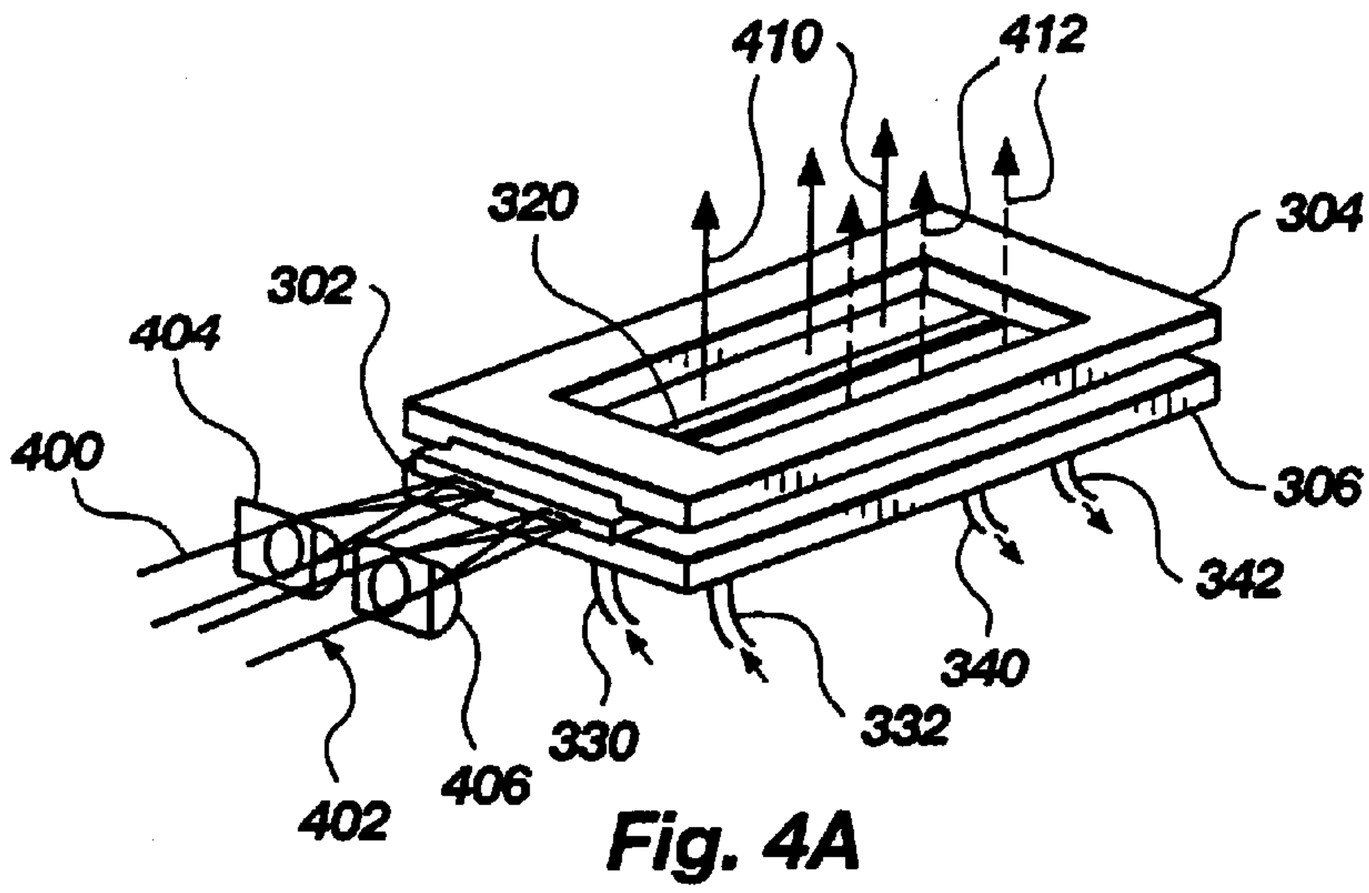


Fig. 4A

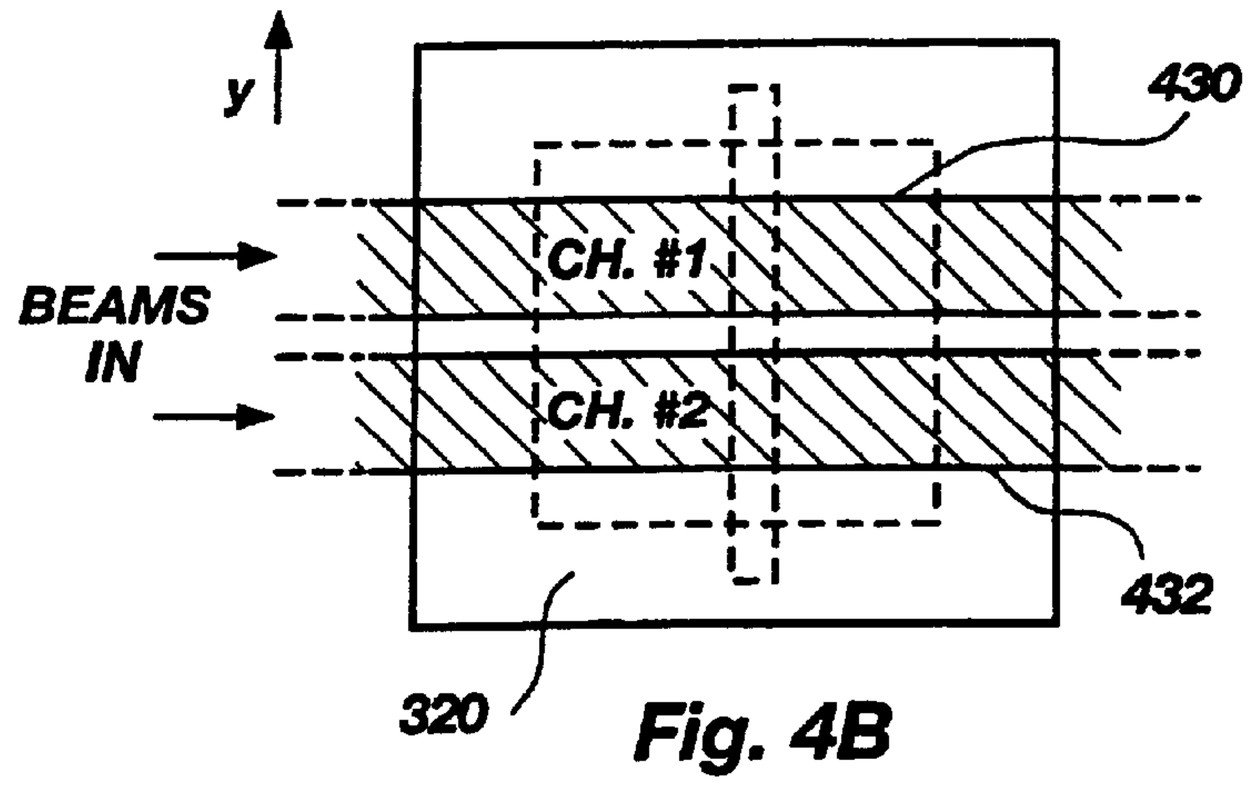


Fig. 4B

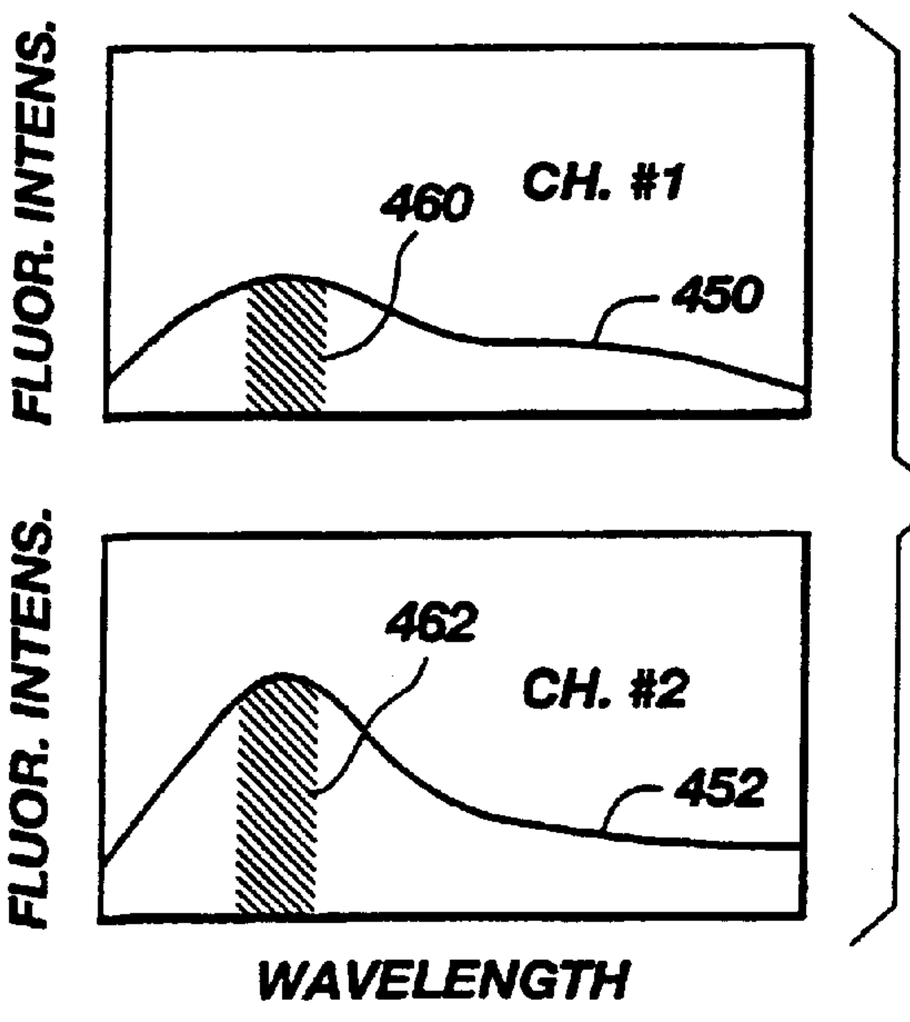


Fig. 4C

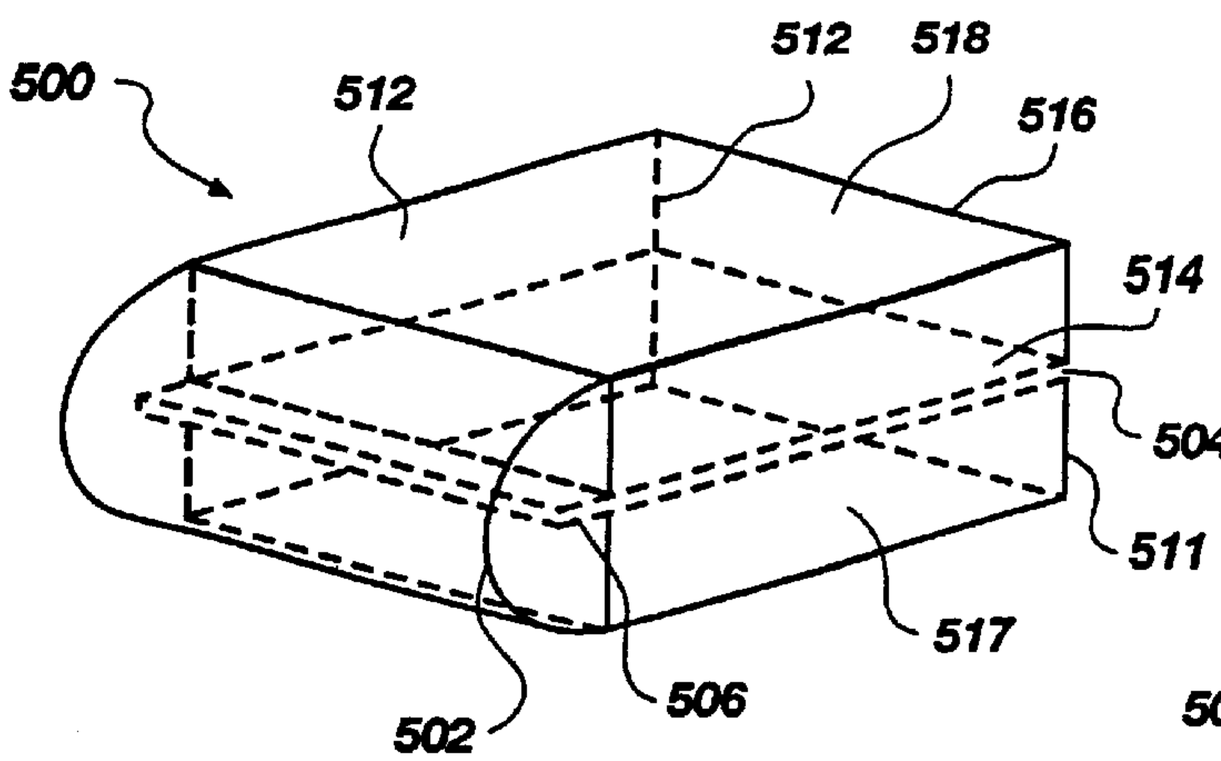


Fig. 5A

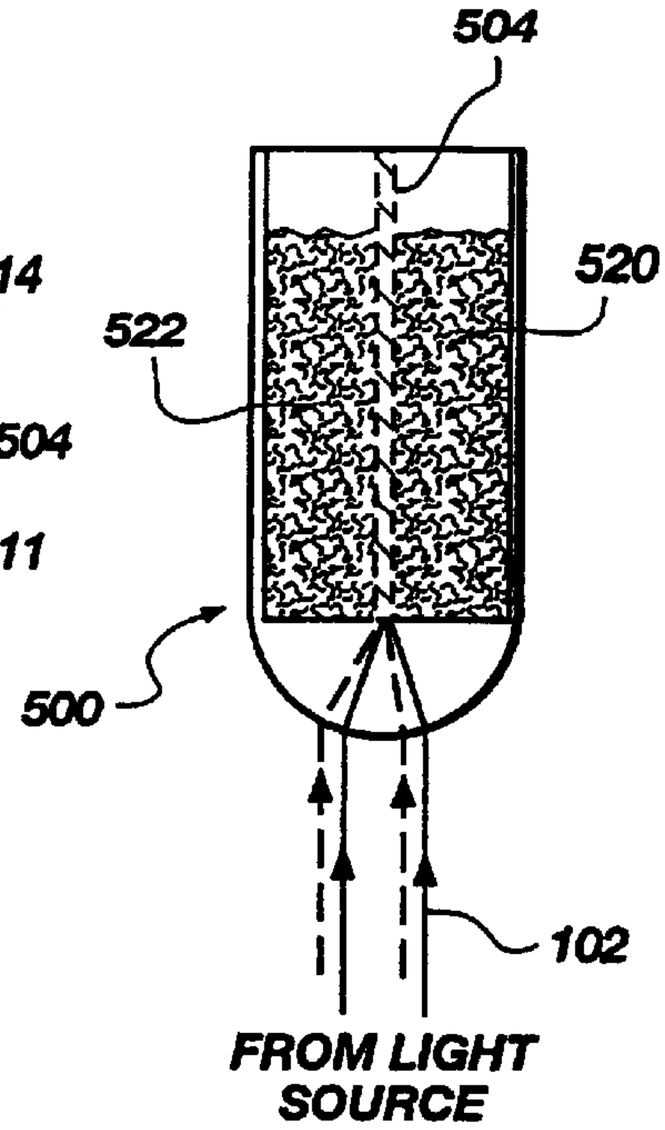


Fig. 5C

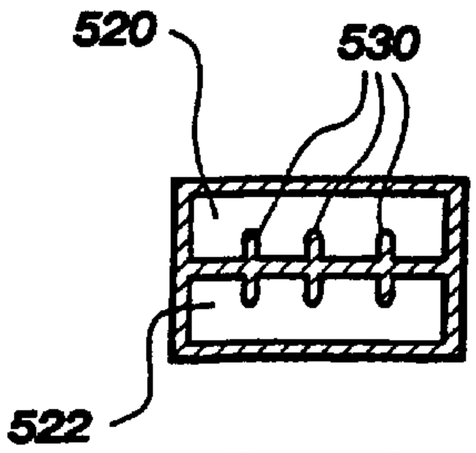


Fig. 5D

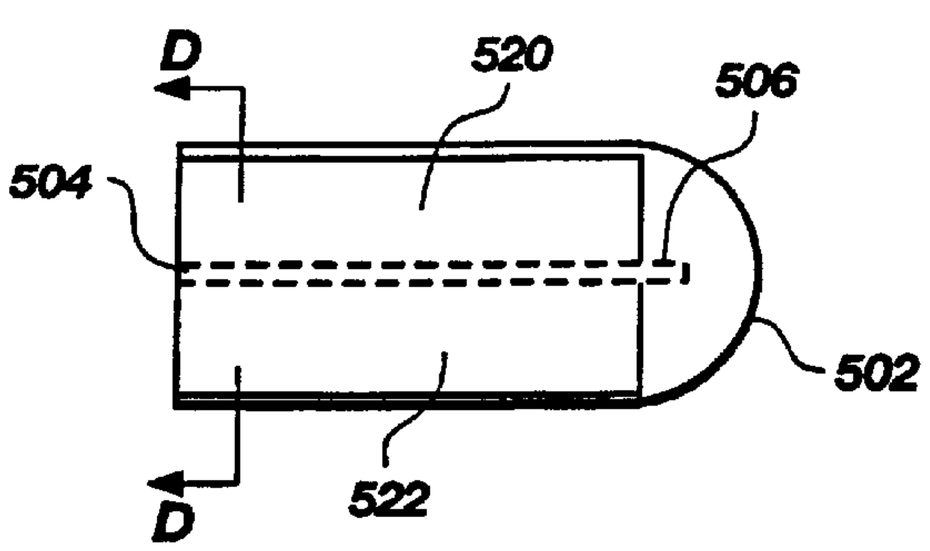


Fig. 5B

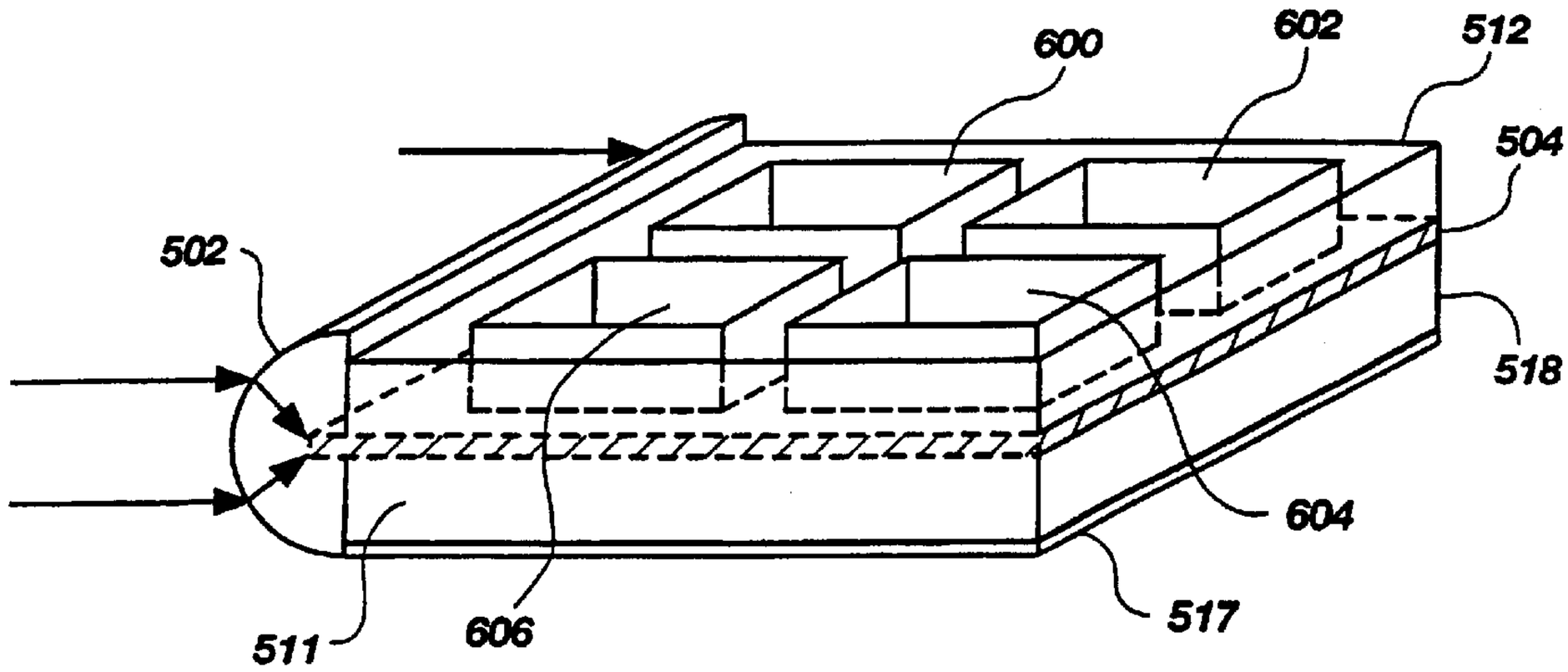


Fig. 6

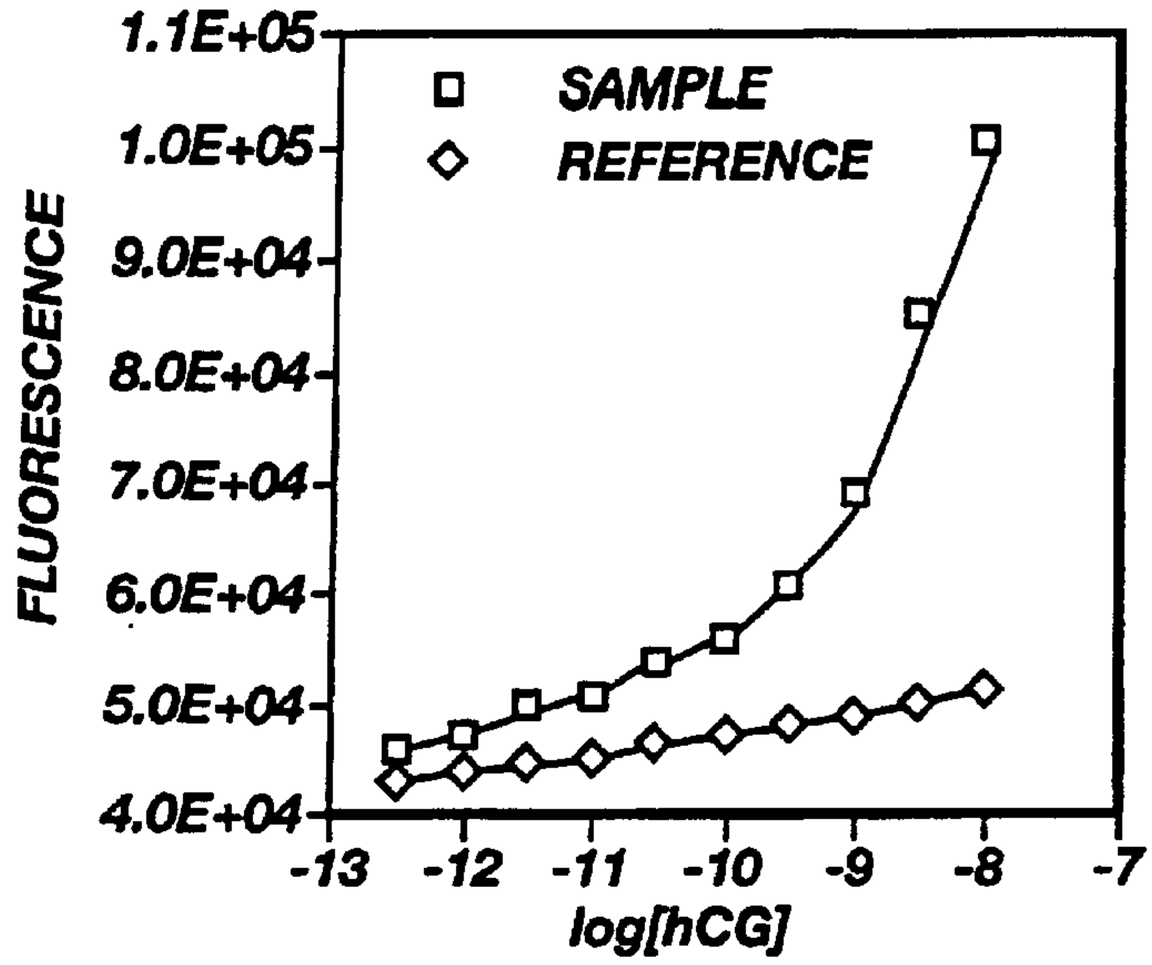


Fig. 7A

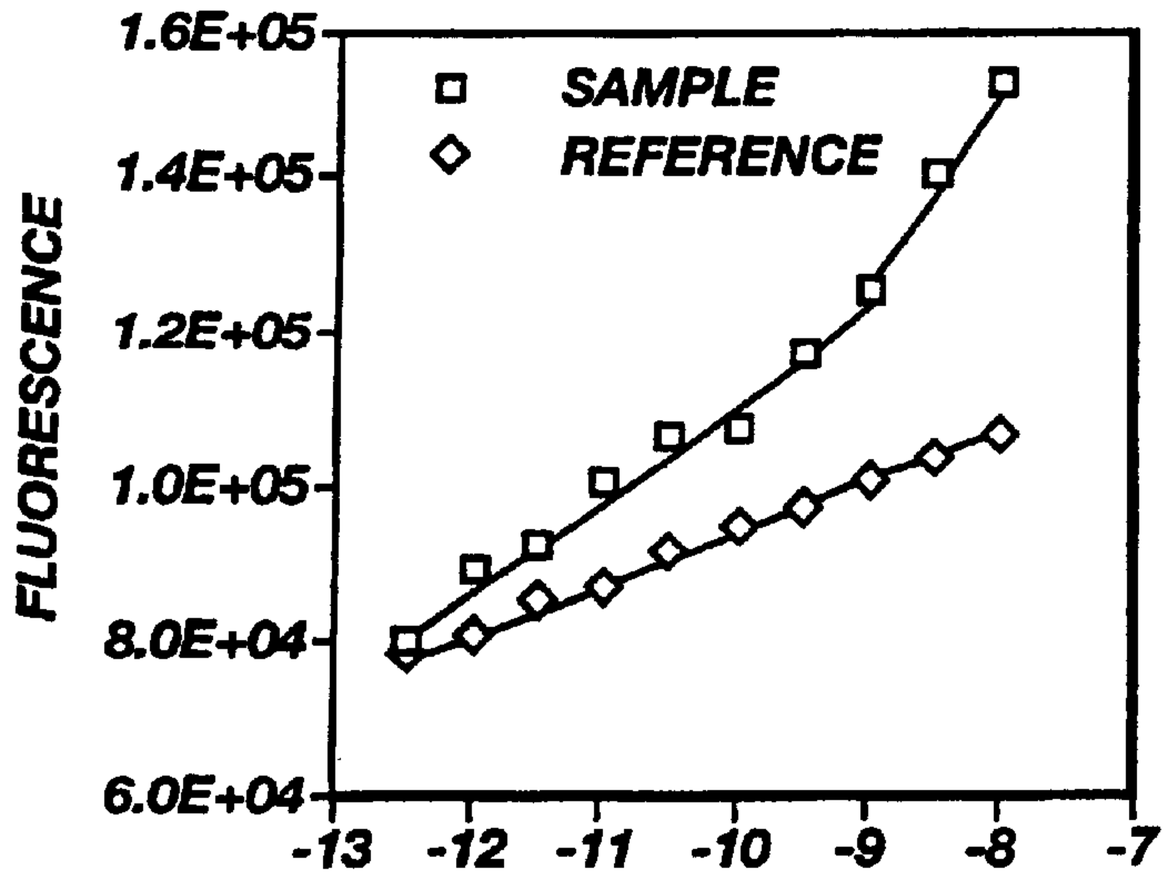


Fig. 7B

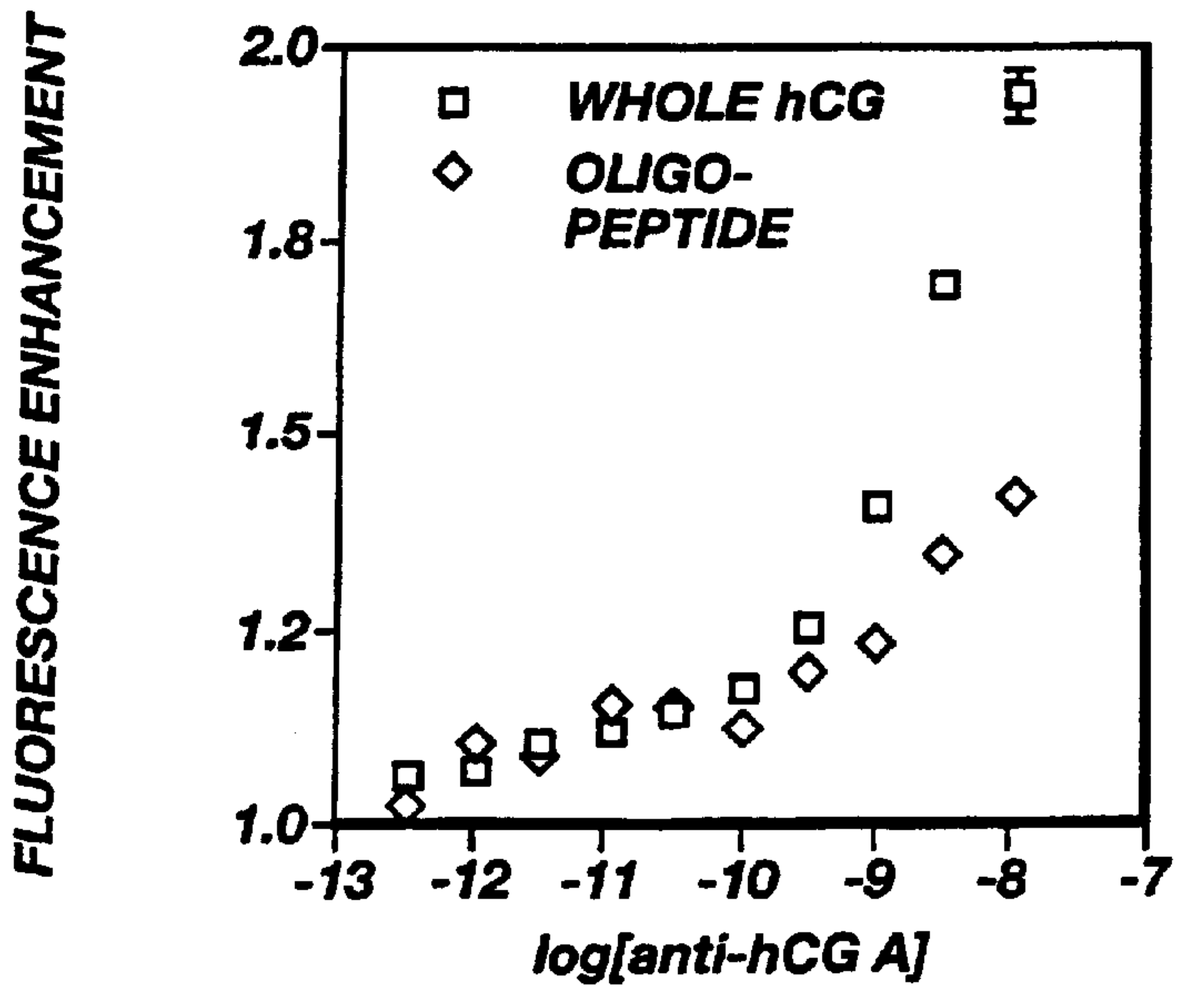


Fig. 8

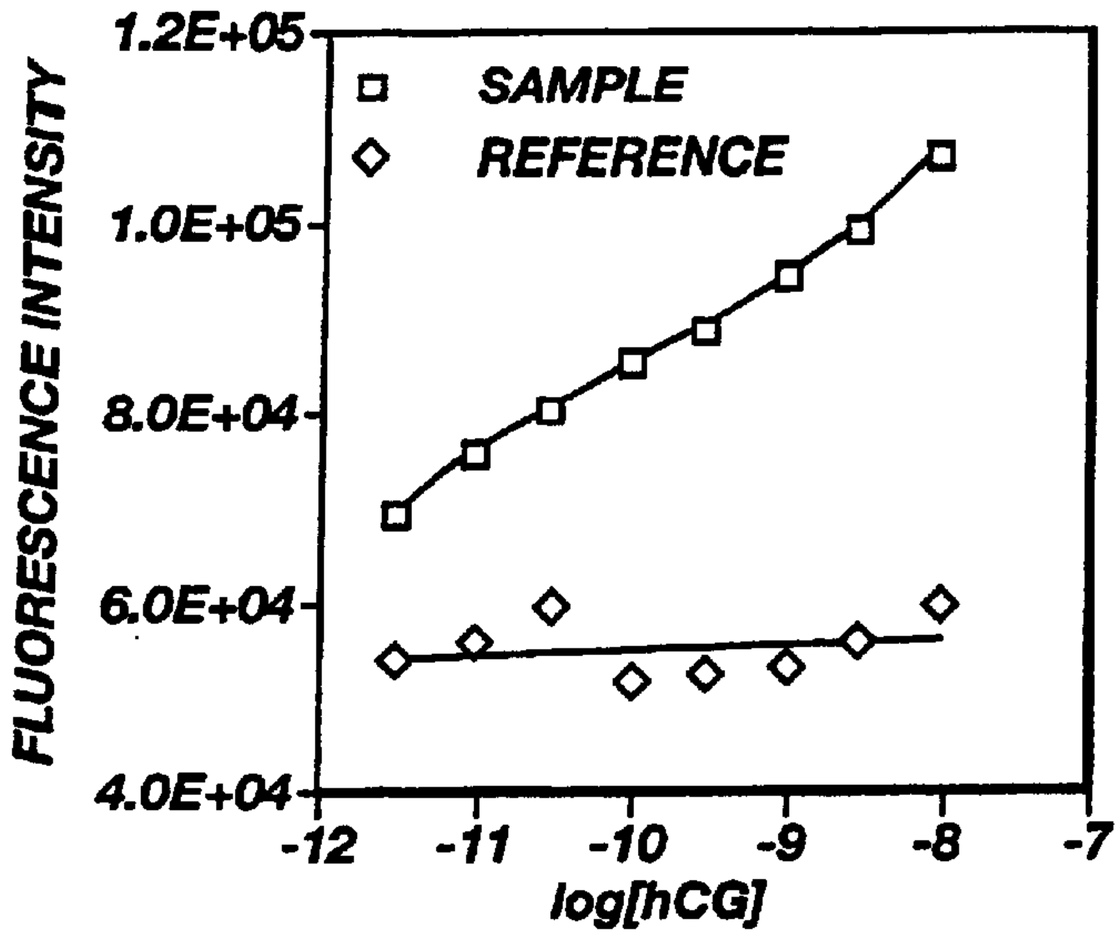


Fig. 9A

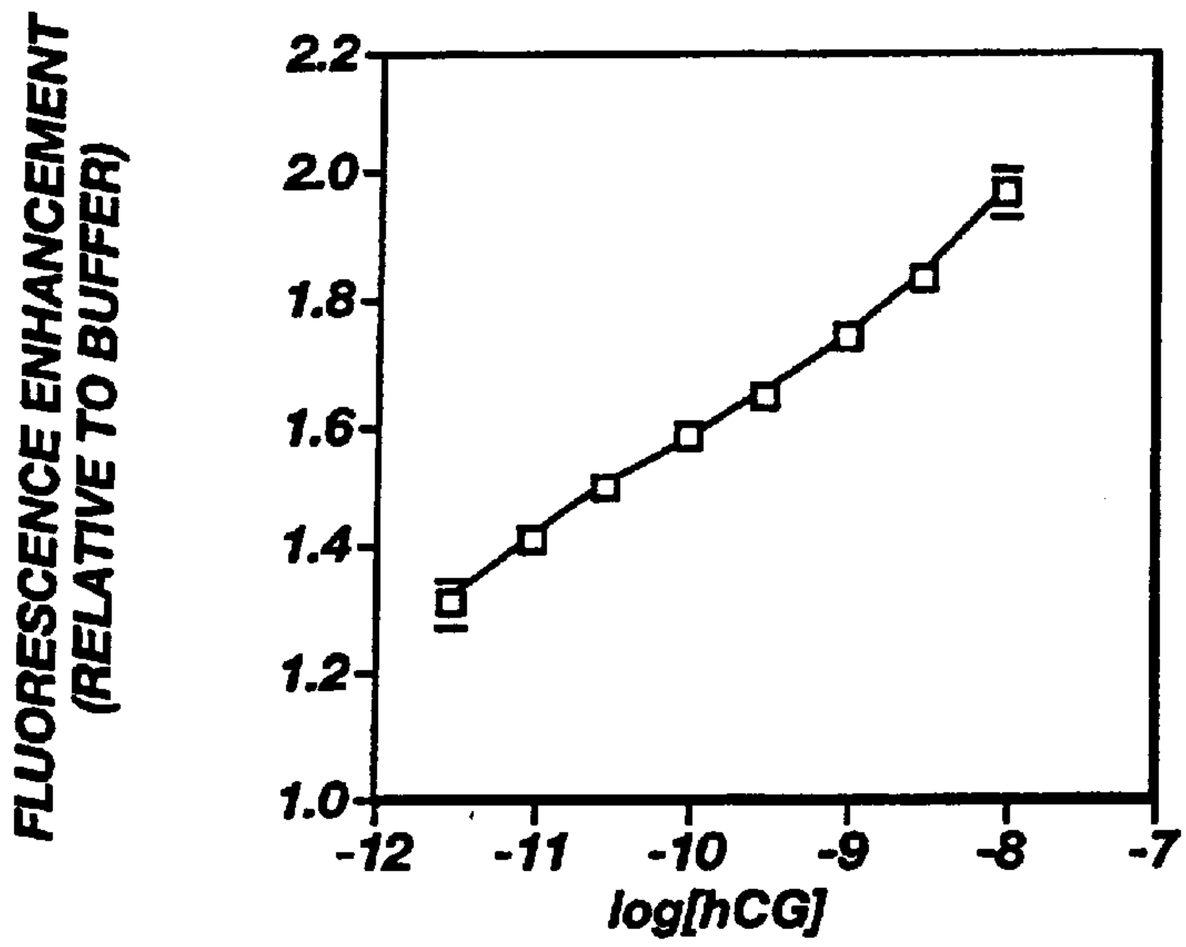


Fig. 9B

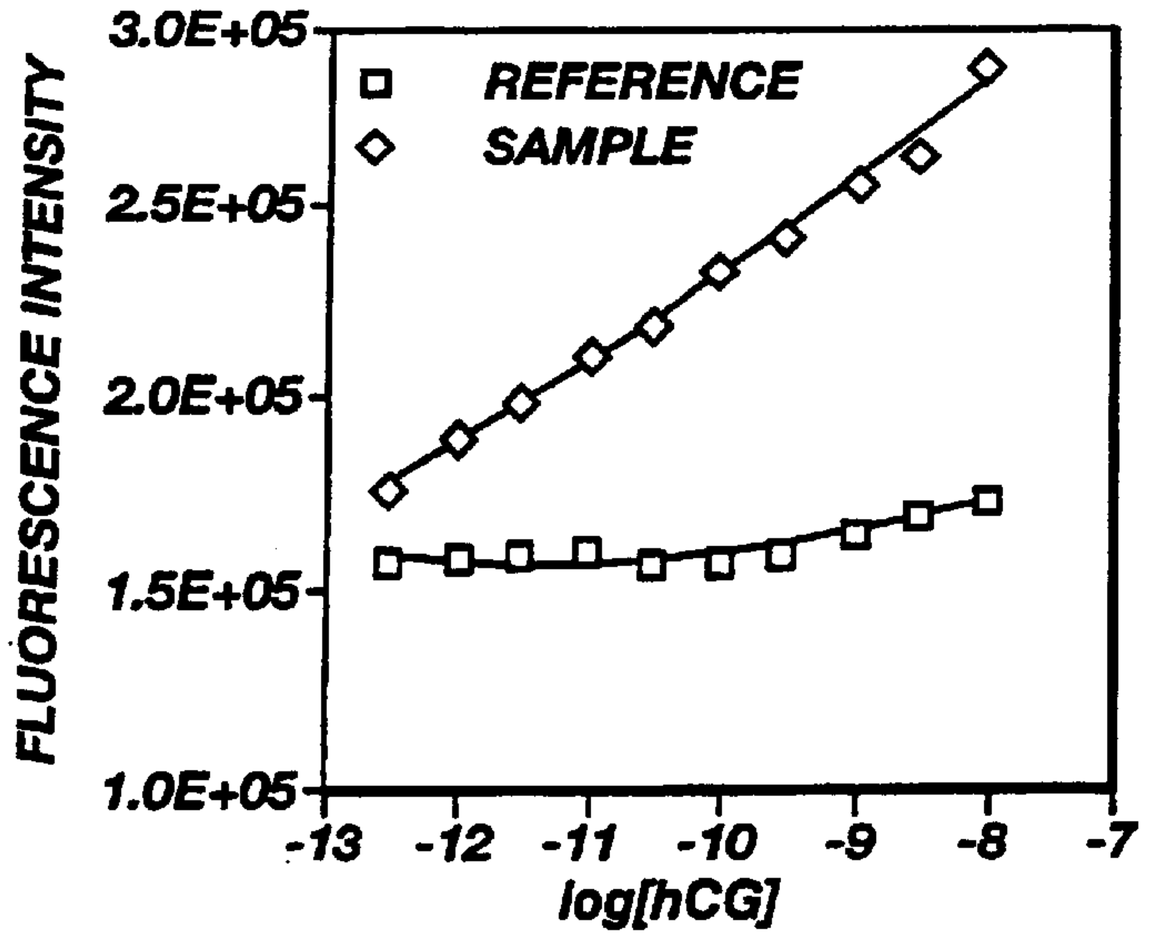


Fig. 9C

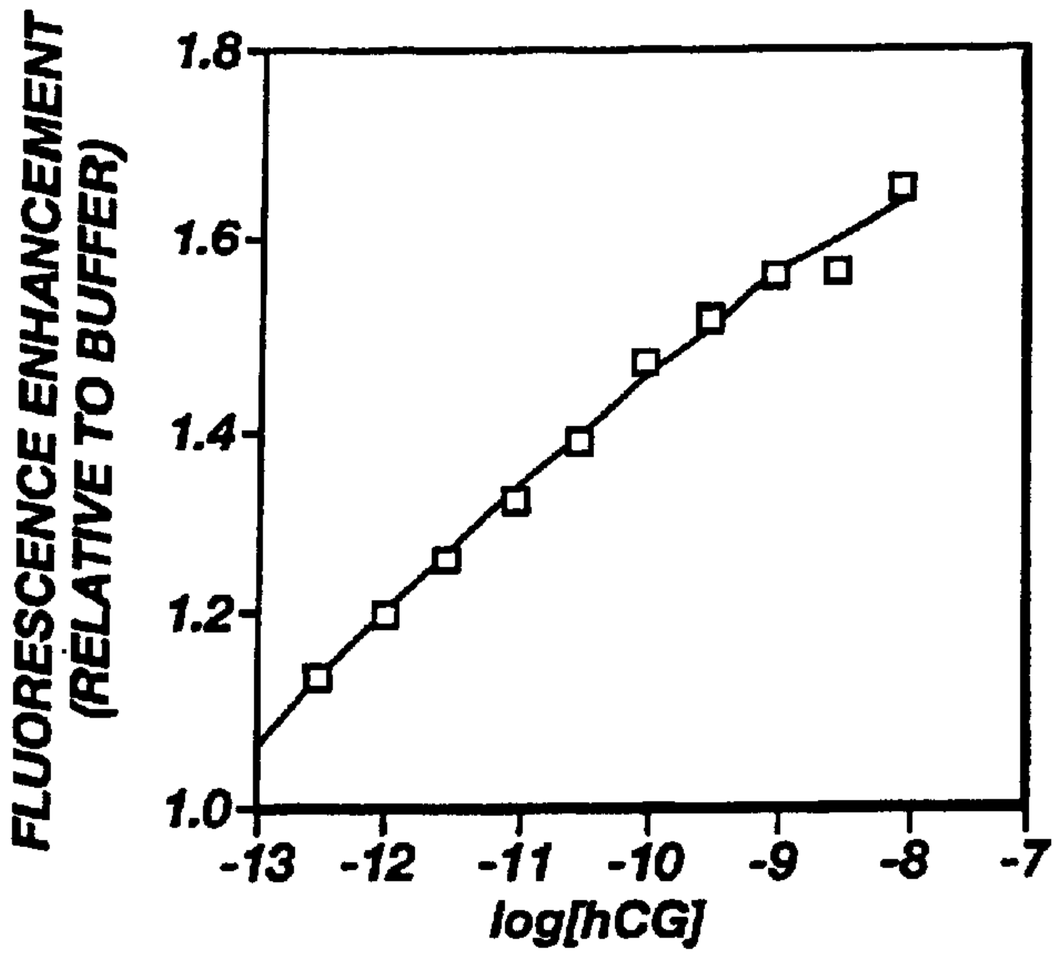


Fig. 9D

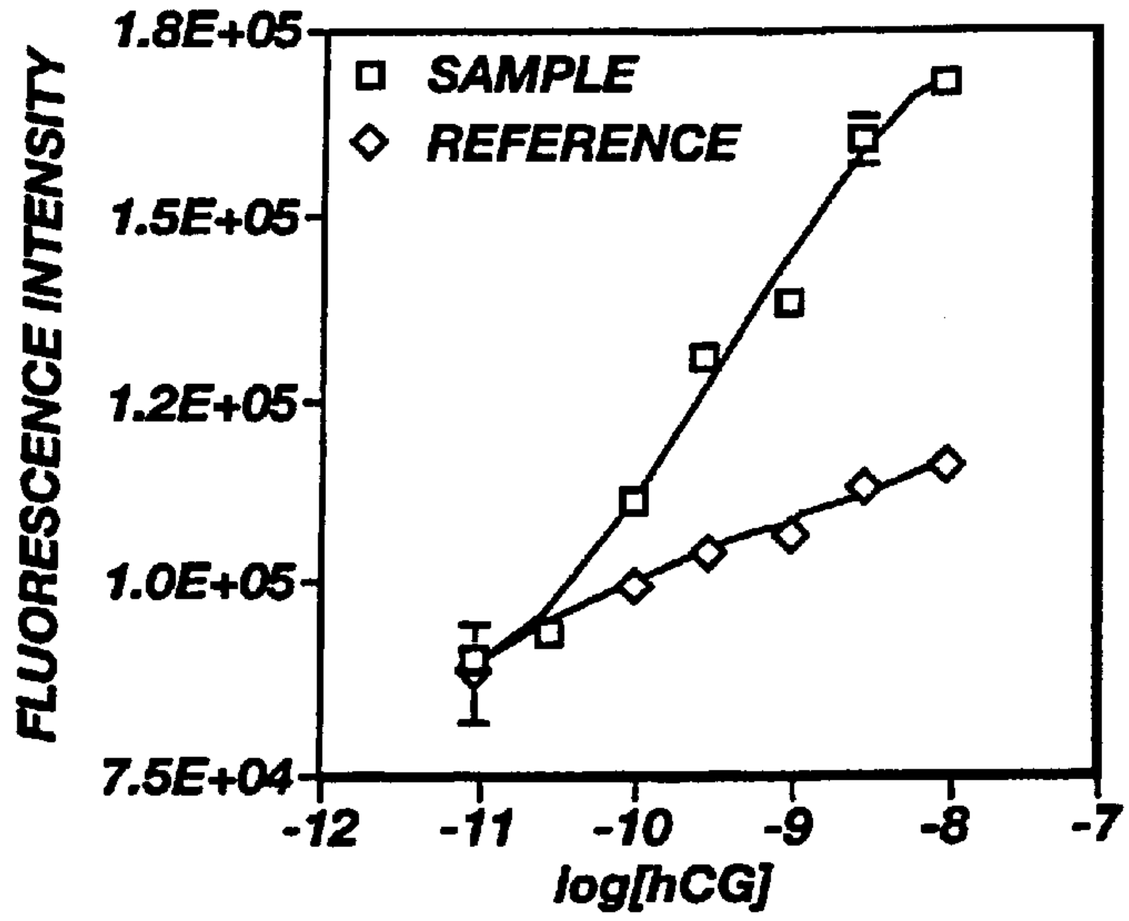


Fig. 9E

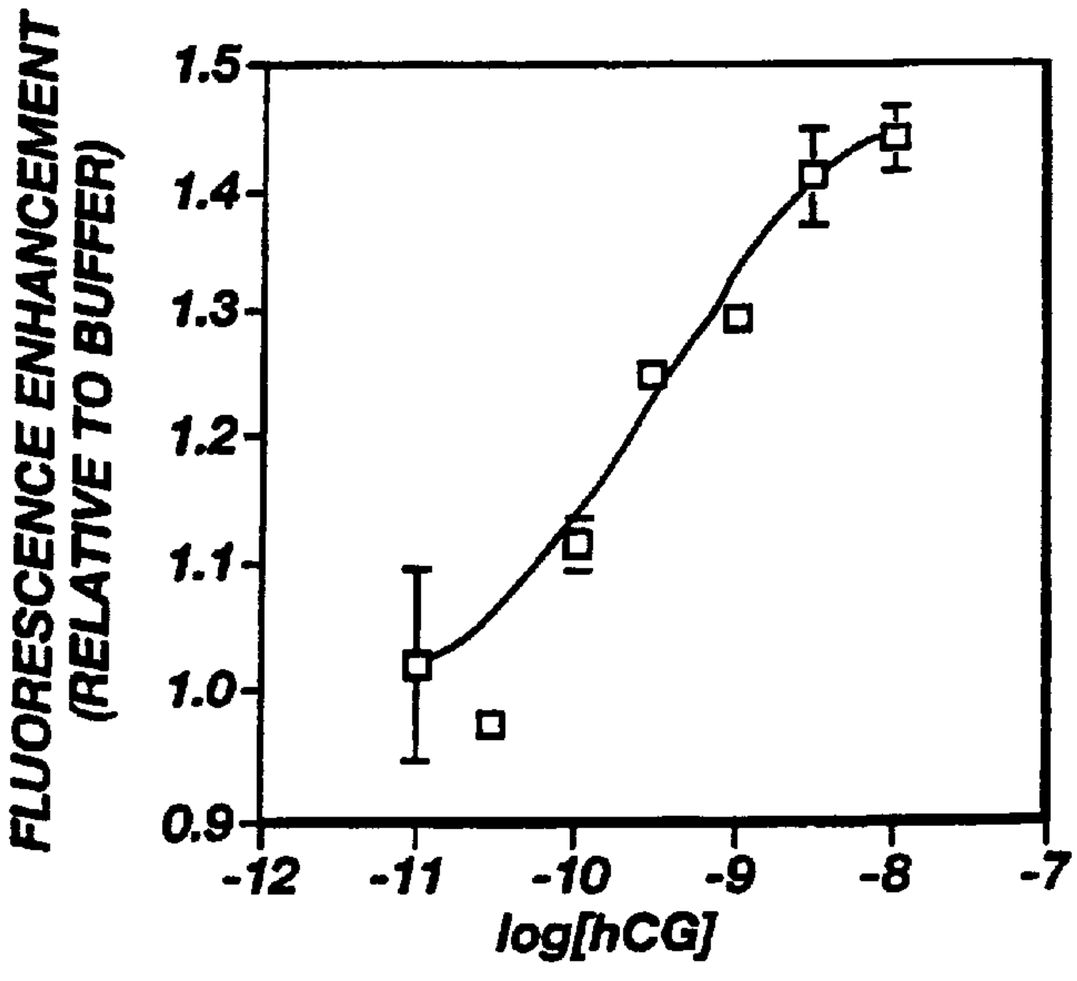


Fig. 9F

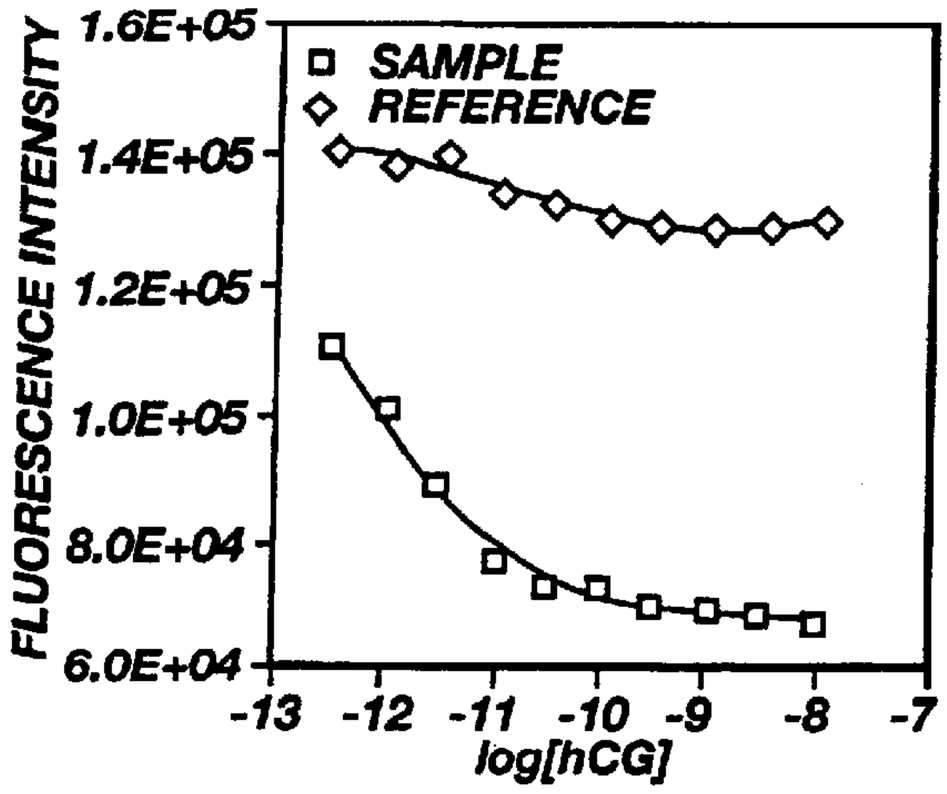


Fig. 10A

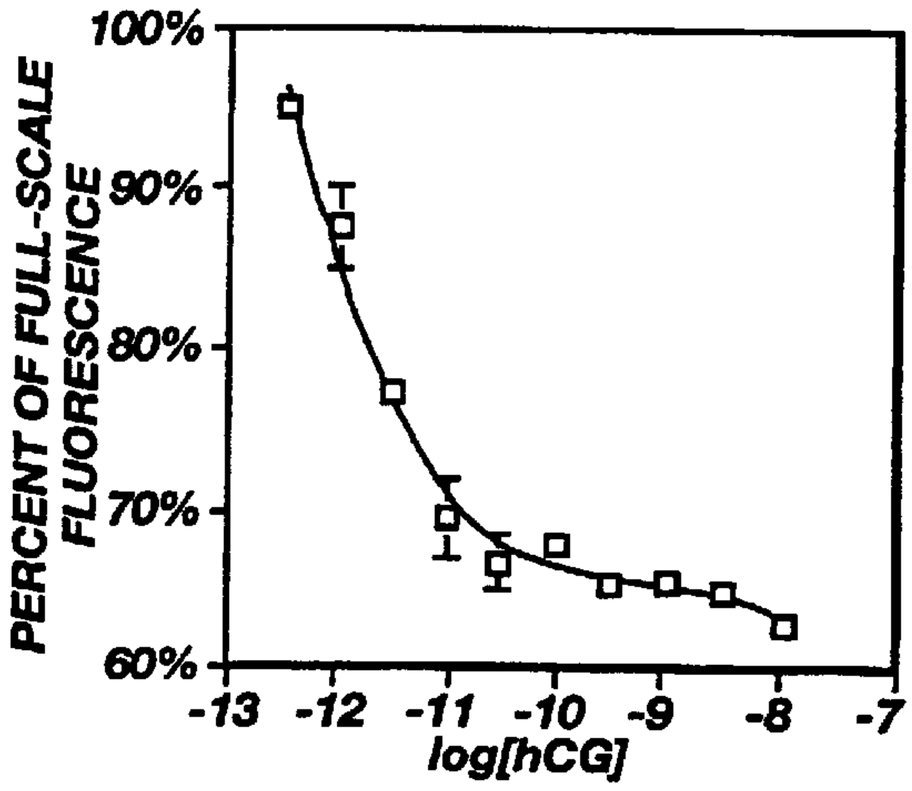


Fig. 10B

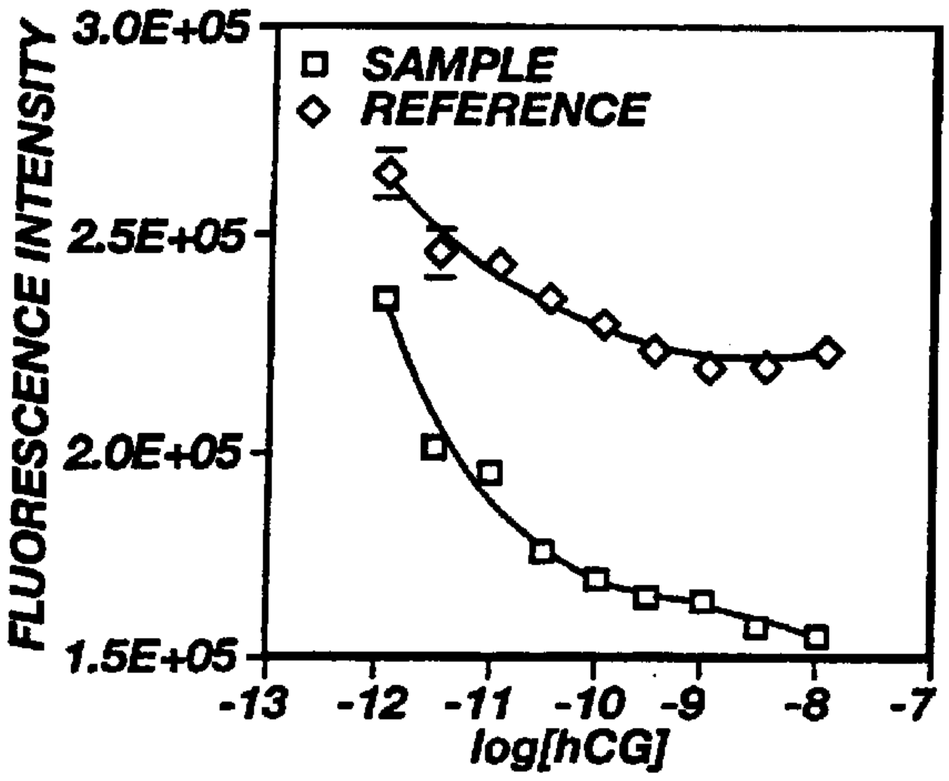


Fig. 10C

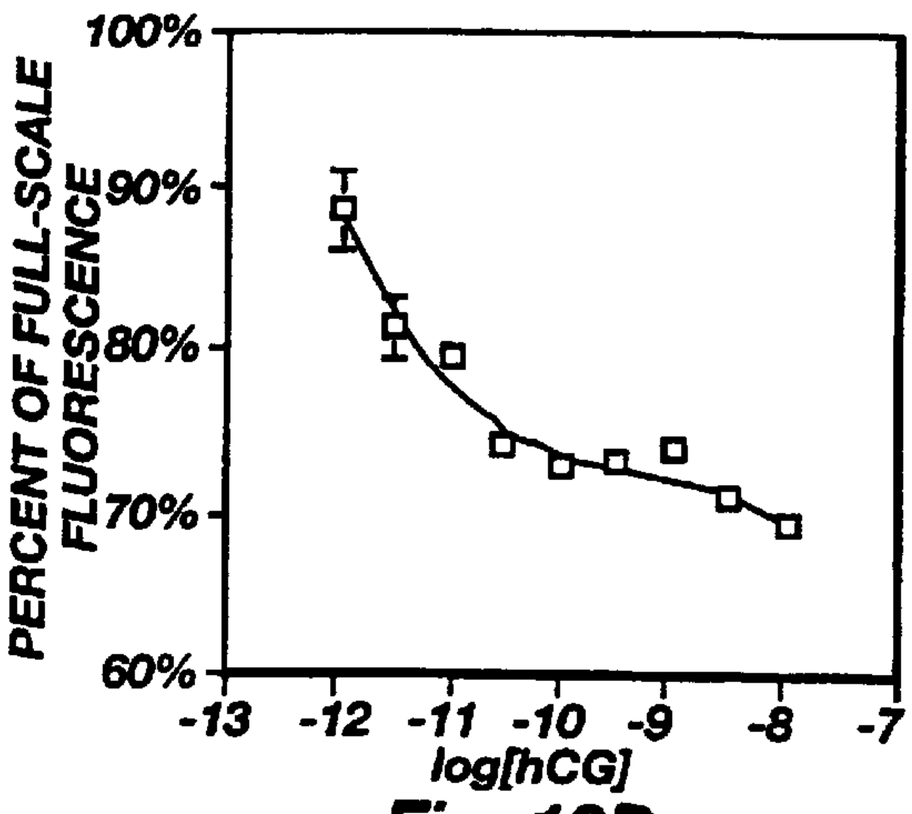


Fig. 10D

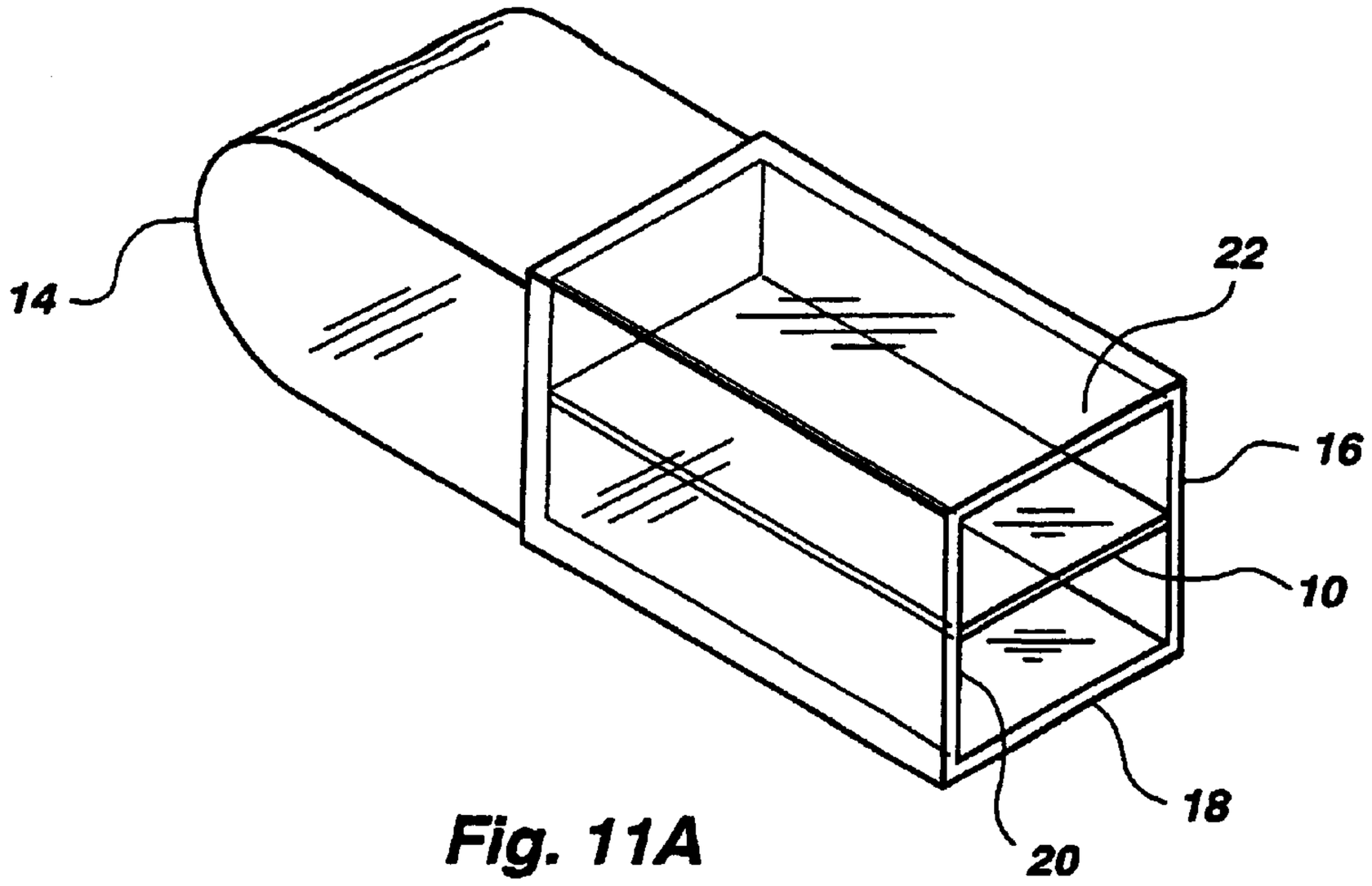


Fig. 11A

Fig. 11B

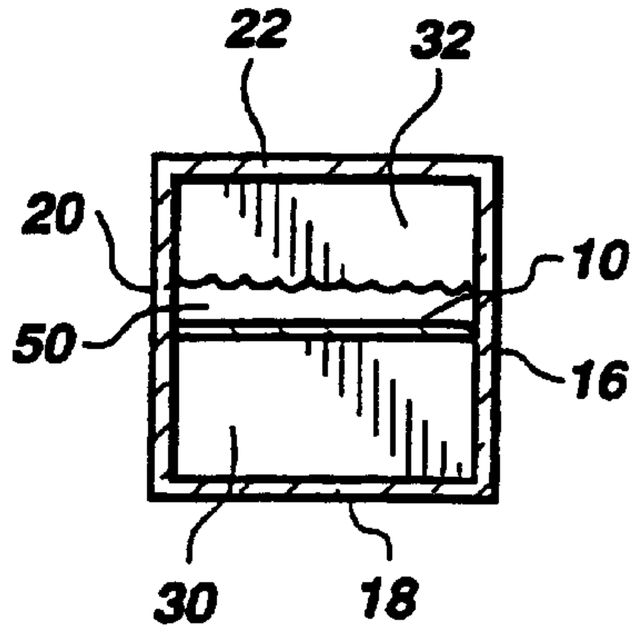


Fig. 11C

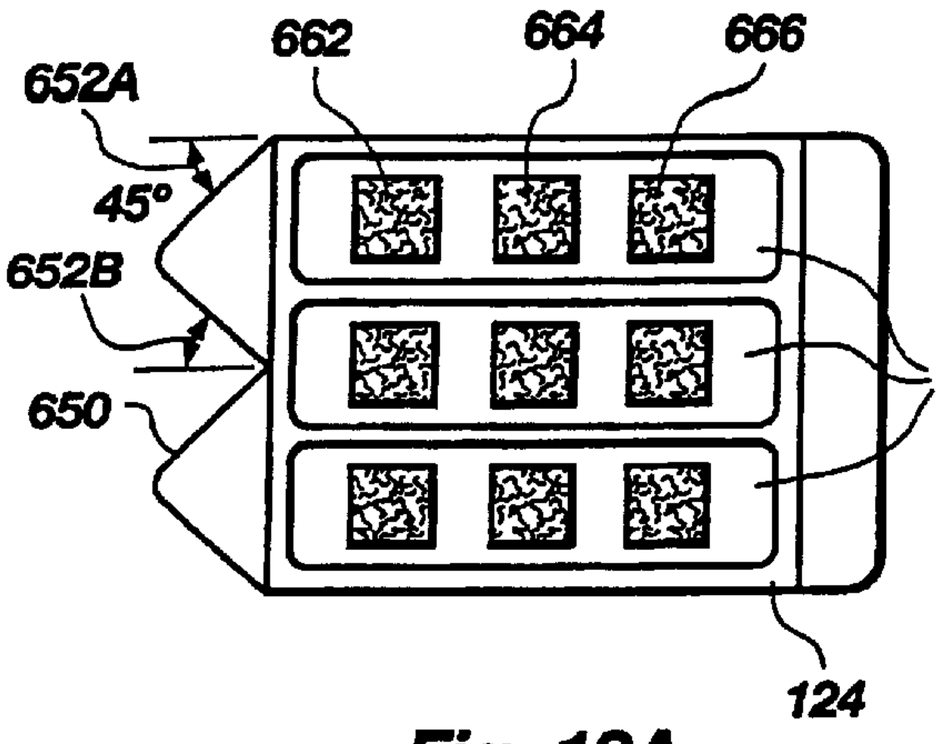


Fig. 12A

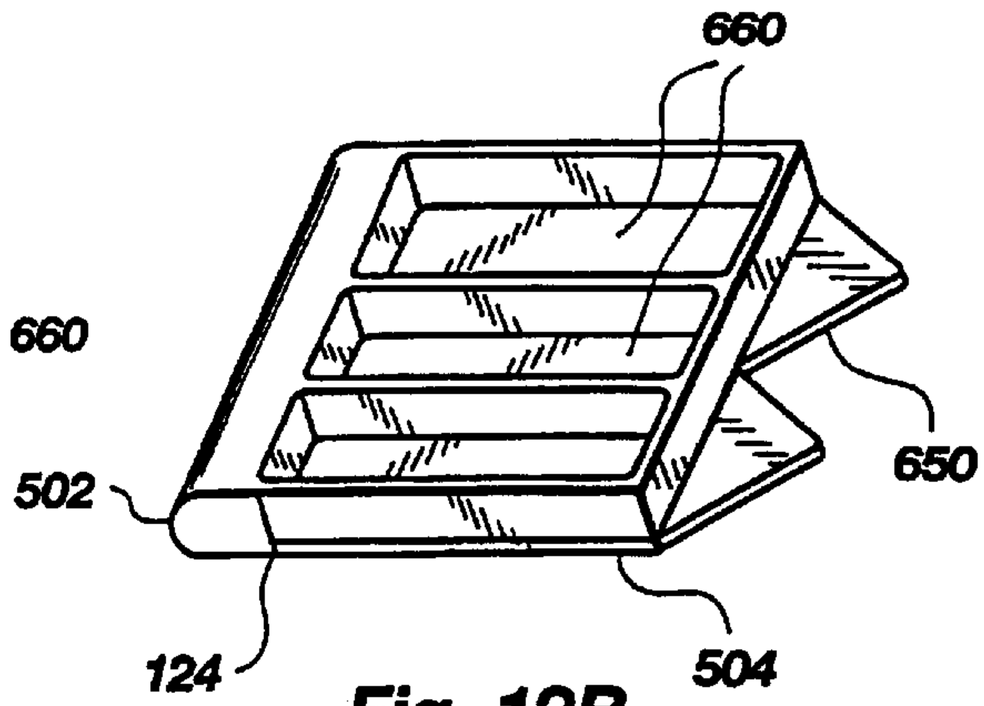


Fig. 12B

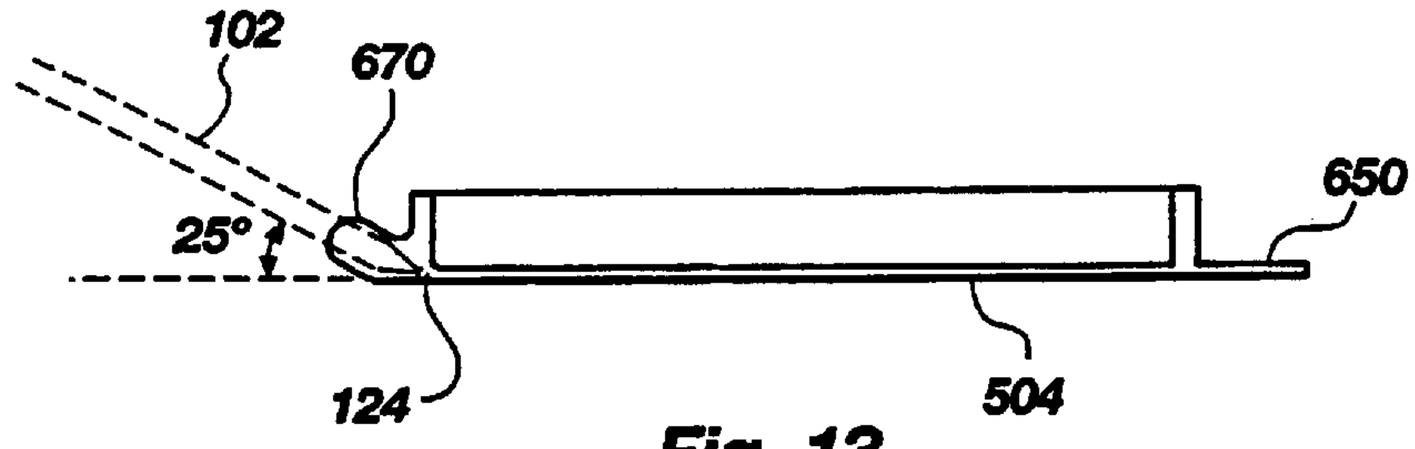
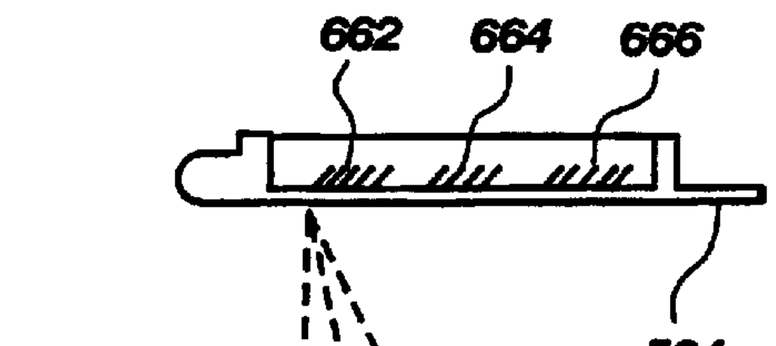


Fig. 13



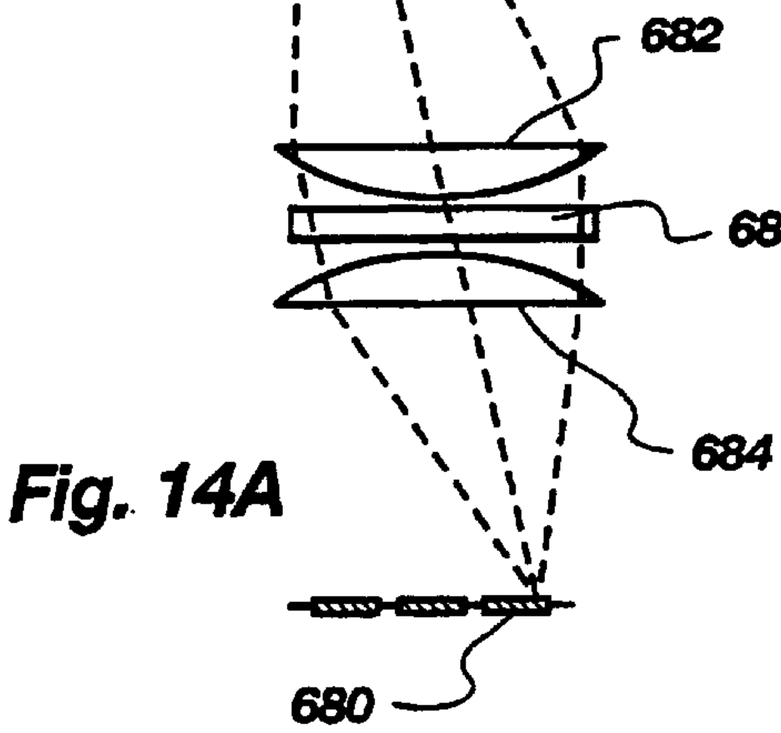


Fig. 14A

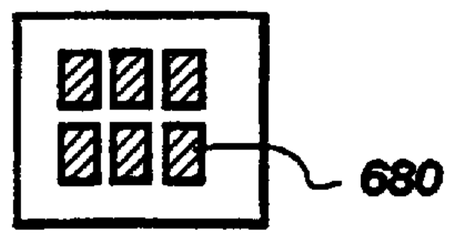


Fig. 14B

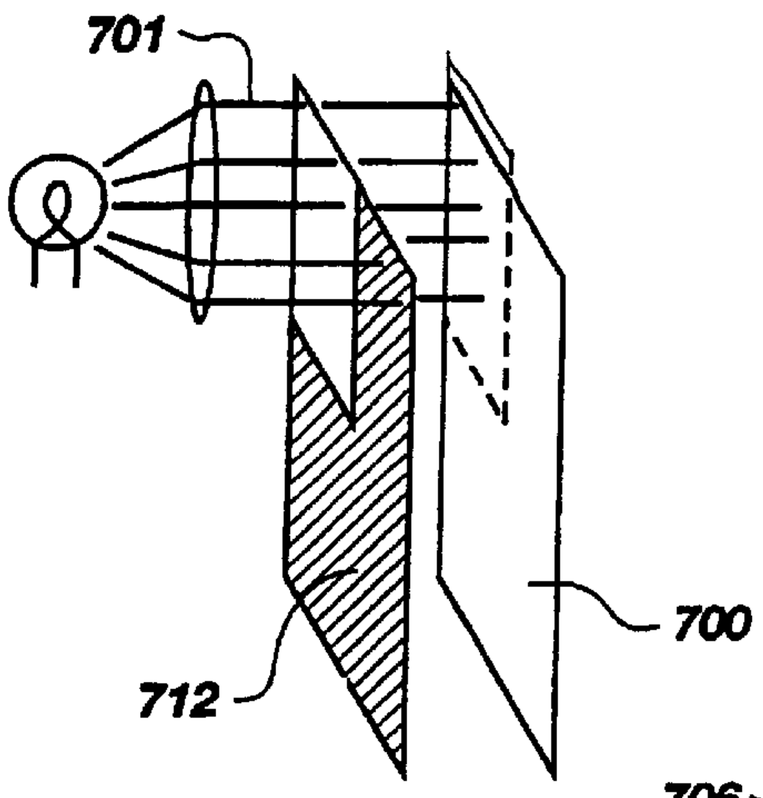


Fig. 15

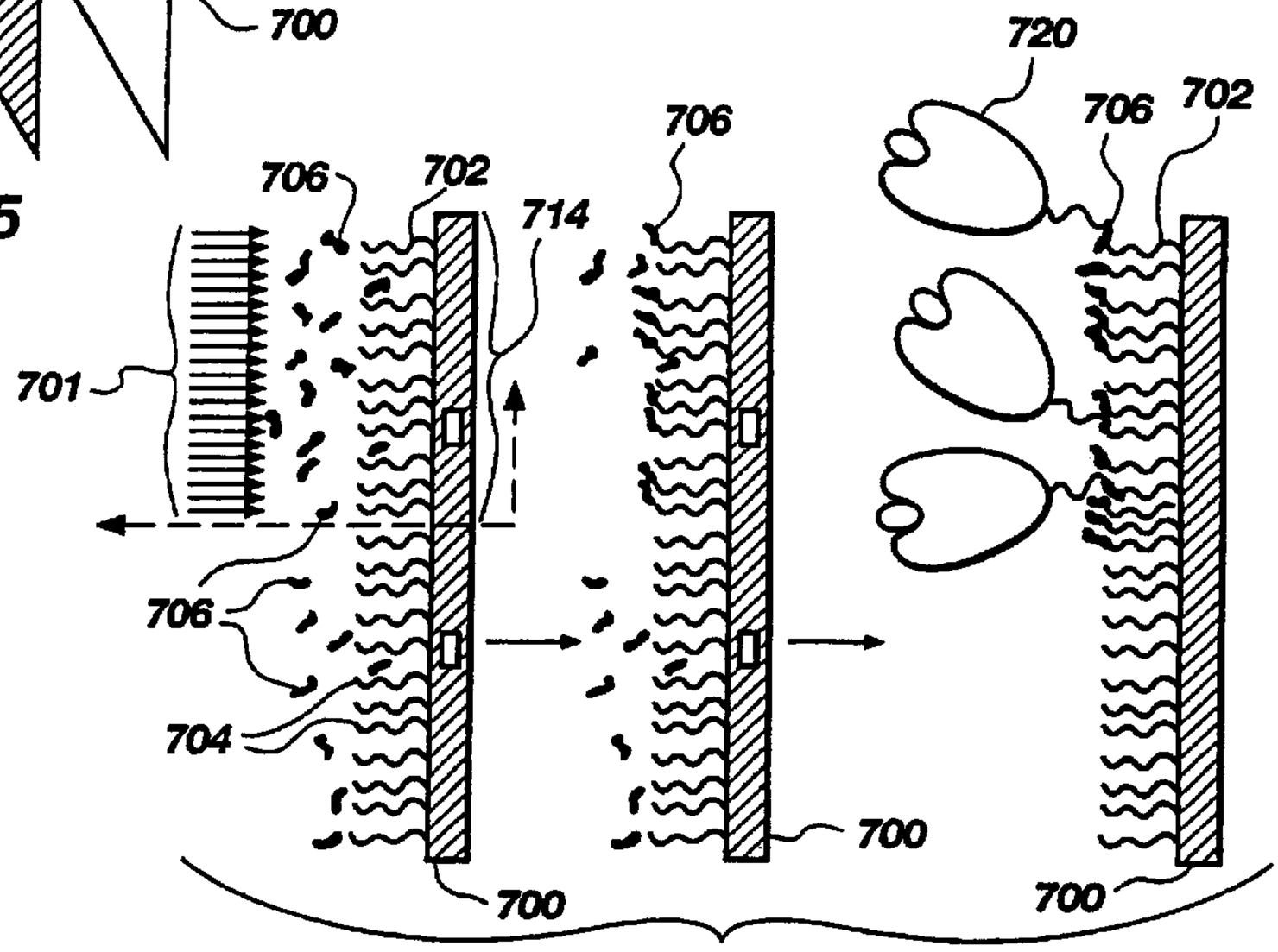


Fig. 16

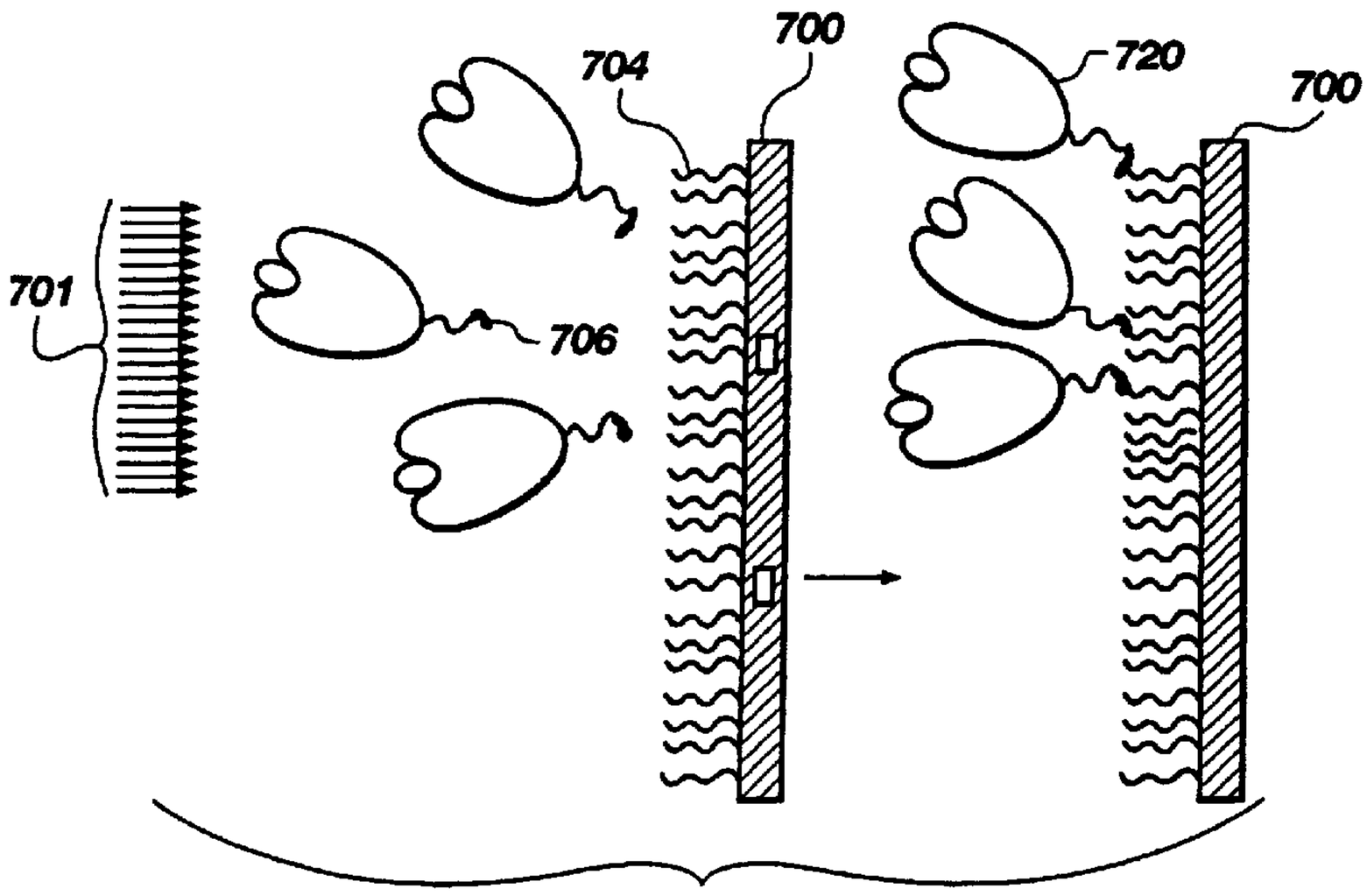


Fig. 17

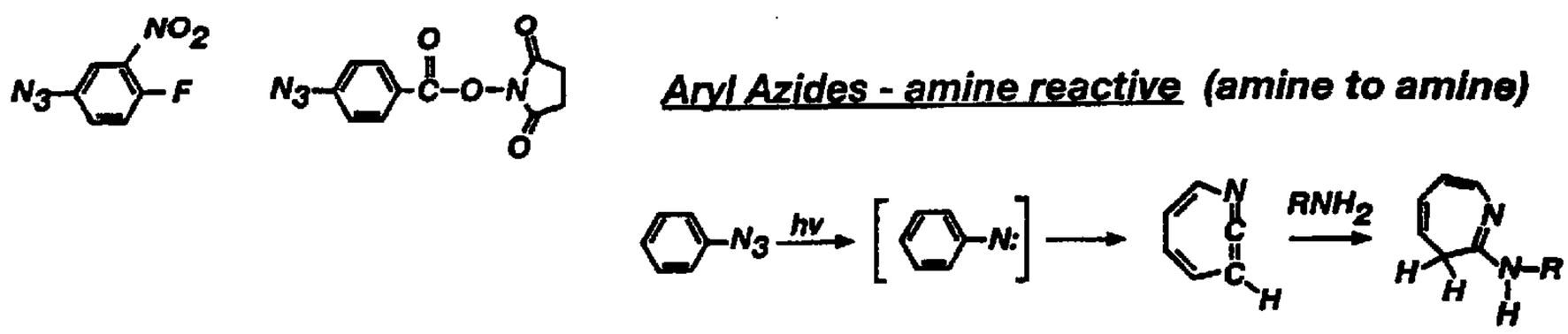


Fig. 18A

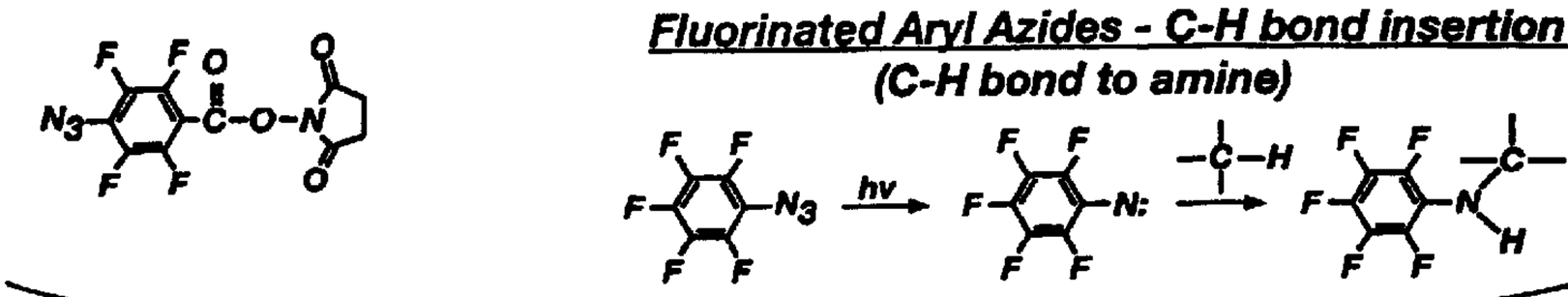


Fig. 18B

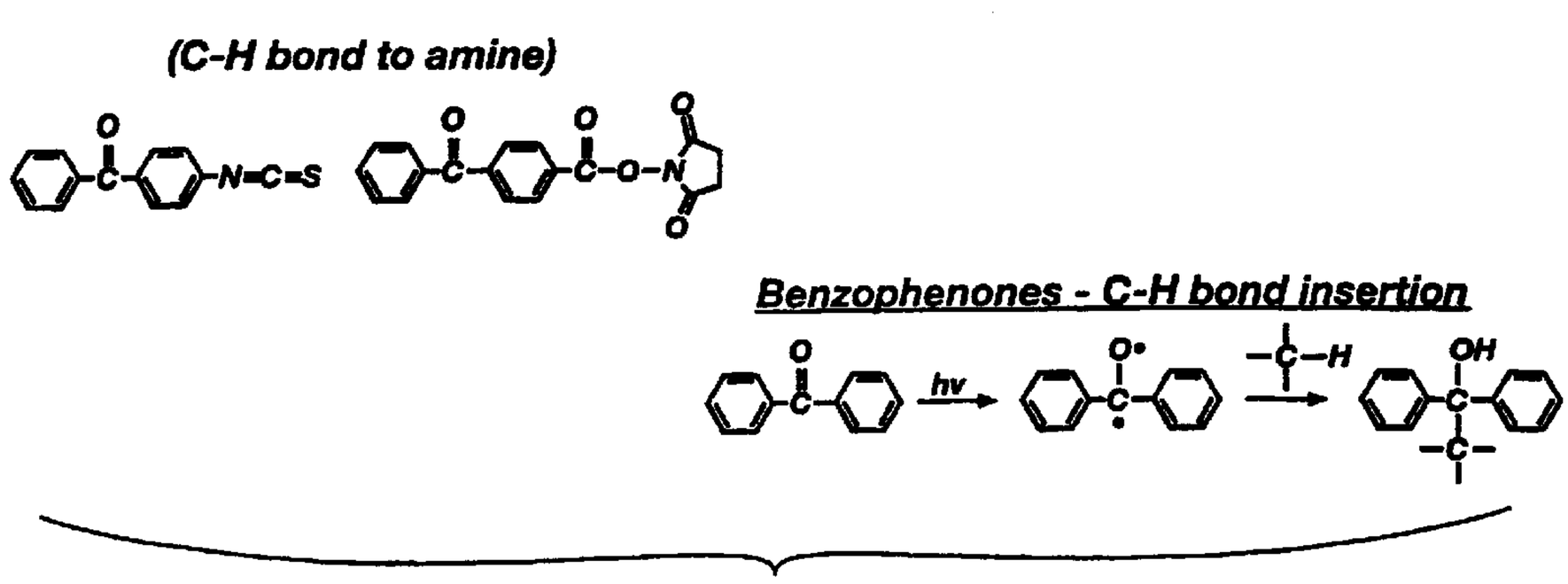


Fig. 18C

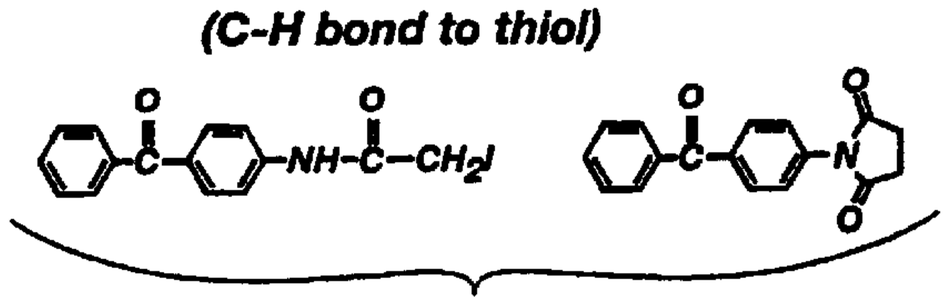


Fig. 18D

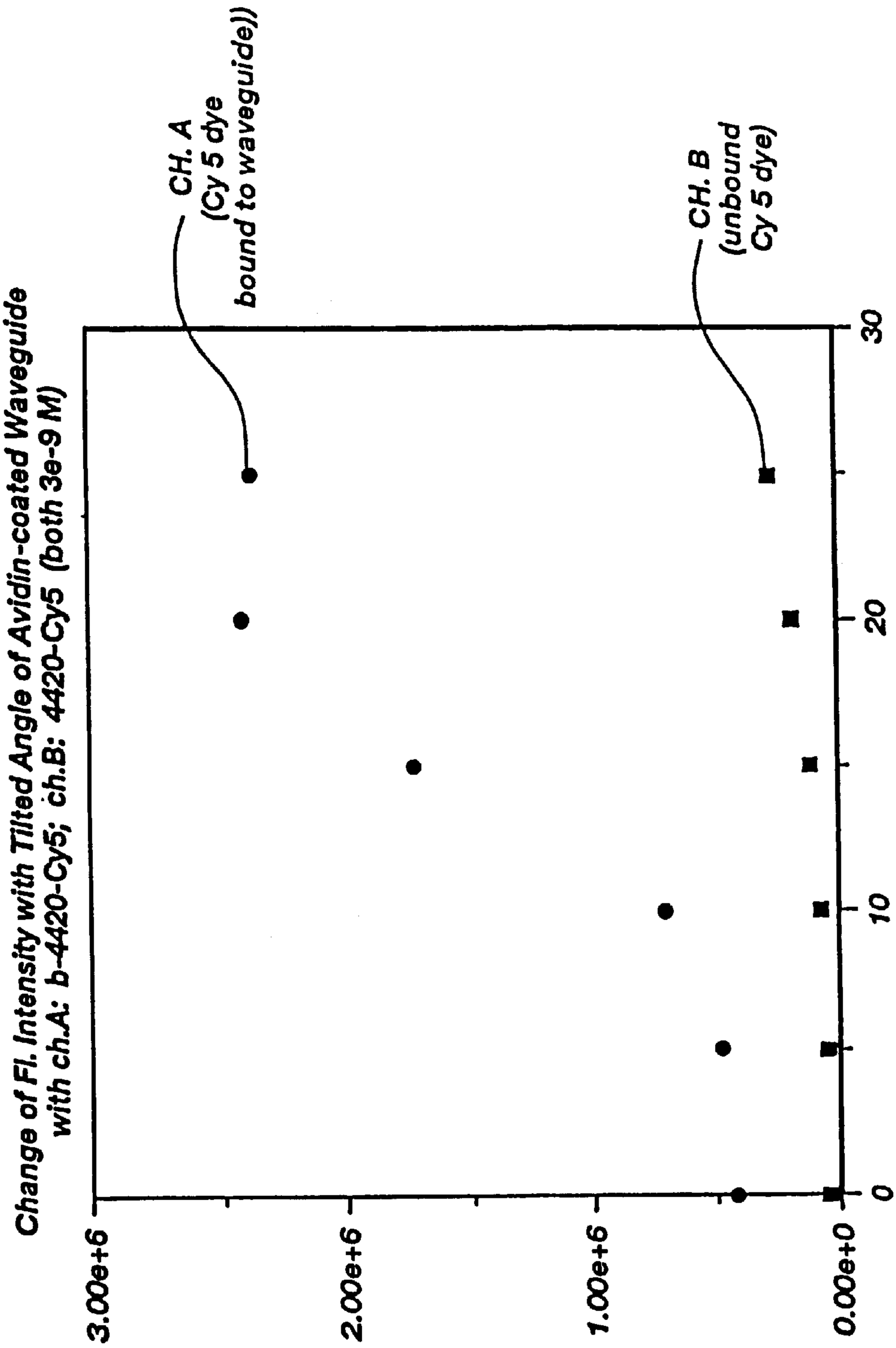

Change of Fl. Intensity with Tilted Angle of Avidin-coated Waveguide
with ch.A: b-4420-Cy5; ch.B: 4420-Cy5 (both 3e-9 M)
CH. A
(Cy 5 dye
bound to waveguide))
CH. B
(unbound
Cy 5 dye)
3.00e+6
2.00e+6
1.00e+6
0.00e+0
FLUORESCENCE INTENSITY
0
10
20
30
BEAM ENTRY ANGLE (DEGREES)

Fig. 19

APPARATUS AND METHODS FOR MULTI-ANALYTE HOMOGENEOUS FLUORO-IMMUNOASSAYS

RELATED APPLICATIONS

This application is a division of application Ser. No. 08/979,582 filed on Nov. 26, 1997, to be abandoned, which is a continuation of U.S. patent application Ser. No. 08/748,687, filed on Nov. 13, 1996, now U.S. Pat. No. 5,919,712, which is a divisional of Ser. No. 08/263,522, filed Jun. 22, 1994, now U.S. Pat. No. 5,677,196, which is a continuation-in-part of application Ser. No. 08/110,169, filed Aug. 20, 1993, now U.S. Pat. No. 5,516,703, and application Ser. No. 08/071,579, filed Jun. 2, 1993, now abandoned, which is a continuation-in-part of application Ser. No. 08/064,608, filed May 18, 1993, now U.S. Pat. No. 5,512,492.

TECHNICAL FIELD

This application relates to the art of analyzing samples for particular substances by means of fluorescent binding assays, and more particularly to apparatus, compositions and methods for such assays employing evanescent light.

BACKGROUND

Biosensor apparatus based on optical detection of analytes by fluorescence of tracer molecules, have attracted increasing attention in recent years. Such apparatus are useful for both diagnostic and research purposes. In particular, biosensors for a solid-phase fluoroimmunoassay, in which an antibody or antibody fragment specific to the desired analyte is immobilized on a substrate, and binding of the analyte to the antibody results either directly or indirectly (for example, by means of a labeled tracer) in a fluorescence signal, are becoming an important class of optical biosensor.

In most solid-phase fluoroimmunoassays, to achieve adequate sensitivity a "wash" step is required to remove unbound tracer before measuring the fluorescence. This problem is particularly true for detection of analytes present at concentrations below nanomolar, as is the case for many analytes of interest in body fluids including blood, serum and urine. However, the wash step is tedious, and care on the part of the technician is required to produce repeatable and accurate results. Accordingly, it is highly desirable to provide a fluoroimmunoassay system in which sensitivity to analyte concentrations of 10^{-10} to 10^{-13} molar or below is achieved without a wash step.

An optical technique known as total internal reflection (abbreviated TIR) provides one approach to such a system. Evanescent light is light produced when a light beam traveling in a waveguide is totally internally reflected at the interface between the waveguide and a surrounding medium having a lower refractive index. A portion of the electromagnetic field of the internally reflected light penetrates into the surrounding medium and constitutes the evanescent light field. The intensity of evanescent light drops off exponentially with distance from the waveguide surface. In a fluoroimmunoassay, evanescent light can be used to selectively excite tracer molecules directly or indirectly bound to an immobilized binding agent, while tracer molecules free in solution beyond the evanescent penetration distance are not excited and thus do not contribute "background" fluorescence. The use of evanescent field properties for fluorescence measurements is sometimes referred to as evanescent sensing. For a glass or a similar silica-based material, or an optical plastic such as polystyrene, with the surrounding

medium being an aqueous solution, the region of effective excitation by evanescent light generally extends about 1000 to 2000 Å (angstroms) from the waveguide surface. This depth is sufficient to excite most of the tracer molecules bound to the capture molecules (antibodies, receptor molecules, and the like, or fragments thereof) on the waveguide surface, without exciting the bulk of the tracer molecules that remain free in solution. The fluorescence thus resulting reflects the amount of tracer bound to the immobilized capture molecules, and in turn the amount of analyte present.

The tracer fluorescent light will conversely also evanescently penetrate back into the waveguide and be propagated therein. The maximum solution depth for efficient evanescent collection by the waveguide approximates the depth of the region of evanescent penetration into the solution, and thus the waveguide-penetrating portion of the tracer fluorescence can also be used to selectively measure fluorescence from tracer bound to the waveguide surface.

U.S. Pat. No. RE 33,064 to Carter, U.S. Pat. No. 5,081,012 to Flanagan et al, U.S. Pat. No. 4,880,752 to Keck, U.S. Pat. No. 5,166,515 to Attridge, and U.S. Pat. No. 5,156,976 to Slovacek and Love, and EP publications Nos. 0 517 516 and 0 519 623, both by Slovacek et al, all disclose apparatus for fluoroimmunoassays utilizing evanescent sensing principles.

Desirably, an immunofluorescent biosensor should be capable of detecting analyte molecules at concentrations of 10^{-12} M (molar) or below. To date, most reports of evanescent-type biosensors indicate that at best, concentrations of 10^{-11} M could be detected.

It is further desirable for speed and convenience in "routine" testing, for example testing of blood bank samples for viral antibodies, to have an evanescent immunofluorescent biosensor which is disposable and which provides multi-sample measurement capability. Multi-sample capability would allow a test sample and a control sample (such as a blank, a positive control, or for a competition-type assay, a sample preloaded with tracer molecules) to be simultaneously illuminated and measured. Simultaneous multi-sample capability would also speed up the process of analyzing multiple samples and would reduce the effects of variation in the level of exciting light which are known to occur with typical light sources. However, in a typical prior art evanescent light device such as that of Block et al, U.S. Pat. No. 4,909,990 issued Mar. 20, 1990, the waveguide is a fiber optic rod whose shape makes it difficult to build a multi-well biosensor.

Another factor which affects the attainable sensitivity relates to the intensity of excitation light emitted from the waveguide. The intensity of fluorescence emitted by tracer molecules is in part dependent on the intensity of exciting light (which is the evanescent field). Therefore, increased evanescent light intensity should provide increased fluorescence which in turn would improve the detection sensitivity. The level of evanescent light is in turn dependent on the intensity of the light beam propagating in the waveguide, and this can be increased by decreasing the cross-sectional area of the waveguide.

Previous methods of immobilizing antibodies to optical substrates in evanescent biosensors also present some problems causing reduction in sensitivity. Many such methods utilize the ϵ-amino groups of lysine residues in the protein. This approach has at least two significant disadvantages due to the fact that most proteins have multiple lysine residues. First, the presence of multiple potential coupling sites

(multiple lysine residues) results in multiple random orientations of antibodies on the substrate surface. If the substrate-coupled lysine residue is near the N-terminal of the antibody molecule, the antibody's antigen binding site (which is near the N-terminal) may be effectively unavailable for binding of the analyte.

Second, if multiple lysines on the same antibody molecule are coupled to the substrate, the molecule may be subjected to conformational strains which distort the antigen binding site and alter its binding efficiency. For capture molecules immobilized by typical prior methods, generally only 20% or less of the binding sites are functional for analyte binding. Thus, it is desirable to have a site-specific method for coupling of the antibodies or other proteins, so that the capture molecules will be uniformly oriented and available for analyte binding.

Another problem relates to the levels of non-specific binding to the antibody-coated surface of the optical substrate. These levels are often sufficiently high to make detection of analyte at concentrations below about 10^{-10} M very difficult. Nonspecific binding can be reduced by including a wash step after the sample is incubated with the coated substrate, to remove unbound tracer molecules. However, as discussed above, a wash step is undesirable. Second, non-specific binding can be a serious problem unless the surface is "passivated" with a masking agent such as bovine serum albumin or with a thin coating of hydrophilic polymer such as poly(ethylene glycol) or poly(methacrylate). Without such passivation (which introduces yet another step into the procedure), non-specific binding can be 50% or more of the specific binding. Even with passivated surfaces, non-specific binding can be sufficient to reduce detection sensitivity and reproducibility.

Thus, a need remains for an evanescent biosensor system which provides the desired sensitivity in a homogeneous assay (homogeneous being defined for purposes of this application as meaning an assay that does not require a wash step). A need further remains for such an apparatus with improved sensitivity for detection of analytes at picomolar concentrations and below. A need also remains for an immunofluorescent assay and biosensor with properties of low non-specific binding and having uniformly oriented capture molecules. A need also remains for such a biosensor and assay system which are inexpensive and readily used by non-skilled persons.

SUMMARY OF THE INVENTION

The invention comprises a system including both apparatus and methods for a homogeneous immunofluorescence assay based on evanescent light principles, capable of detecting one or more analytes at concentrations less than pico-molar. The overall configuration of the apparatus is such that fluorescence-emitting tracer molecules bound to a waveguide surface are excited by an evanescent field penetrating into the adjacent solution from a light beam propagated within the waveguide, the propagated beam being introduced at an end or edge of the waveguide. The emitted fluorescence is then directly collected from the zone of evanescent penetration, e.g. not from an edge or end of the waveguide.

The apparatus includes a biosensor comprising a planar waveguide having a receiving region on its edge for receiving light to be internally propagated. A semi-cylindrical lens is integrally adapted to the waveguide edge adjacent the receiving region, and at least one of the waveguide surfaces has a plurality of capture molecules immobilized thereon. The capture molecules may include a plurality of species each configured to specifically bind a different analyte, and different species may be localized in different and mutually exclusive regions on the waveguide surface. In a highly preferred embodiment, the semi-cylindrical lens and the waveguide are integrally molded of an optical plastic, and the lens is oriented to aim the beam at a selected angle to the plane of the waveguide, the selected angle being less than the critical angle of reflection at the waveguide-liquid interface. The waveguide also may have a serrated portion forming the portion of the edge opposite the receiving region.

The apparatus further includes a light source configured and disposed to deliver a sheet beam of light into the waveguide through a receiving region on the edge, and detection means disposed for direct collection of fluorescence from the evanescent zone, direct collection being defined as not requiring propagation of the fluorescent light in the waveguide. The detection means is desirably an imaging detector configured to simultaneously separately collect a plurality of fluorescence signals each originating from a different region on the waveguide surface. The imaging detector includes a plurality of photodetection elements spaced from each other in the displaced parallel plane, and lens means positioned to focus each of said fluorescence signals onto a respective one of the photodetection elements.

The invention further encompasses methods for site-specifically immobilizing the capture molecules to the waveguide surface, methods for coating silica and polystyrene waveguide surfaces to reduce nonspecific binding, methods of patterning a waveguide surface with patches of different capture molecule species using photo-affinity crosslinking agents, and waveguides prepared by any of these methods singly or in combination. The method for patterning the waveguide with patches of different capture molecules involves localized irradiation of one or more regions of the waveguide surface in the presence of a photo-affinity crosslinking agent. In a highly preferred method, the waveguide surface is coated with a coating agent which inhibits non-specific protein binding to less that 5%–10%, and preferably as low as 1% to 2%, of non-specific binding. Also preferably, the capture molecules are coupled to the waveguide in a site-specific manner. For site-specific coupling, Fab' fragments are preferred as these are easily prepared with thiol sites, the thiol sites being highly suitable for the surface coupling chemistry. The preferred method of photo-activated crosslinking agent also utilizes thiol sites on the capture molecules. The site-specific coupling chemistry provides waveguide surfaces having 50% to 70% of the capture molecules with analyte binding sites readily available for binding.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram of a fluorescent immunoassay apparatus of the invention;

FIG. **2** is a side view of a portion of the waveguide and the biochemical components of a competition assay according to the invention;

FIG. **3**A is a top view of a flow biosensor of the apparatus of FIG. **1**;

FIG. **3**B is a side cross-section view of the flow biosensor taken along line B—B in FIG. **3**A;

FIG. **3**C shows the waveguide in isolation as it could be arranged with respect to a cylindrical lens and incoming and reflected light waves;

FIG. **4**A is an elevational view of a two-channel flow biosensor of FIGS. **3**A–**3**B with respect to exciting light beams and the collection of fluorescence;

FIG. **4**B is a schematic diagram of the two-channel biosensor indicating the arrangement of two components of the detection device, a CCD detector and the entrance spectrometer slit, with respect to the waveguide regions;

FIG. **4**C is a diagram of fluorescence intensities as they might be detected from two channels of a biosensor arranged according to FIGS. **4**A and **4**B;

FIG. **5**A is an elevational view of an alternate embodiment of a multichannel biosensor;

FIG. **5**B is a side view of the biosensor of FIG. **5**A;

FIG. **5**C is a side view of the biosensor of FIG. **5**A in a vertical orientation with a sample solution therein;

FIG. **5**D is a cross-sectional view of the biosensor taken along line D—D in FIG. **5**C;

FIG. **6** is an elevational view of an alternate embodiment of a multiwell biosensor;

FIG. **7**A is a chart depicting fluorescence intensity data from a sandwich fluoroimmunoassay for detecting an antibody, and performed with the apparatus of FIG. **1** according to a first assay format;

FIG. **7**B is a chart depicting data from a sandwich fluoroimmunoassay performed with the apparatus of FIG. **1** according to an alternate assay format;

FIG. **8** is a chart comparing the fluorescence enhancement observed with the assay formats of FIGS. **7**A and **7**B;

FIGS. **9**A–F are charts depicting data from an alternate scheme for a sandwich fluoroimmunoassay for detecting an analyte using a corresponding antibody;

FIGS. **10**A–D are charts depicting data from a displacement fluoroimmunoassay performed with the apparatus.

FIG. **11**A is an elevational view of another embodiment of a biosensor;

FIG. **11**B is a side cross-section view of the biosensor embodiment of FIG. **11**A;

FIG. **11**C is an end view of the biosensor embodiment of FIG. **11**A;

FIGS. **12**A and **12**B are a top view and an elevation view, respectively, of an alternate embodiment of an integrally molded biosensor;

FIG. **13** is a side view of a molded biosensor with an alternate embodiment of the integral lens;

FIGS. **14**A and **14**B are a side view diagram of an improved imaging photodetection system and a top view of a photodiode array for use in the system;

FIG. **15** is a perspective diagram of a photo-masking set-up for producing a waveguide with patches of different Fab' species;

FIG. **16** is a side view of a section of the waveguide surface showing in schematic form, steps in a process of patterning a waveguide surface with different Fab' species;

FIG. **17** depicts an alternate embodiment of the patterning process;

FIGS. **18**A, **18**B, **18**C and **18**D show chemical formulas of photo-affinity crosslinkers useful in the invention, and partial chemical reactions for photo-coupling the crosslinkers to a base coating;

FIG. **19** is a chart comparing the intensity of detected fluorescence as a function of the angle of the input light lens to the waveguide surface.

DETAILED DESCRIPTION OF THE ILLUSTRATED EMBODIMENTS

A light source **100** provides a light beam **102** which is directed by means of mirrors **104**, **106**, **108** to an optical biosensor indicated generally at **120** (FIG. **1**). In the working embodiment, light source **100** is an argon laser capable of emitting light at wavelengths of between about 488 and 514.5 nanometers (abbreviated nm). In an alternate embodiment, a laser diode emitting at wavelengths of 600 to about 700 nm can be used as light source **100**. Depending on the requirements of the fluorescent tracer, light source **100** may also be embodied as any other laser or other high-intensity light source emitting a sufficient amount of light at an appropriate wavelength to excite the selected tracer.

The embodiment of FIG. **1** further includes a 45° angle mirror **110** which is positioned for making beam **102** a vertical beam prior to focusing the beam onto the biosensor. It will be understood by those skilled that the number and arrangement of mirrors **104**, **106**, **108**, **110** may be varied as necessary to accommodate various space limitations, with the sole requirement being that a sufficient amount of light be directed to biosensor **120**.

Biosensor **120** has an optical substrate **122** with one end **124** positioned to receive light from beam **102**. A focusing lens **126** is positioned between angle mirror **110** and end **124** of waveguide **122**, for focusing light from beam **102** onto end **124**. Focusing lens **126** is here shown mounted on an X-Y translation unit so that its position may be adjusted for best focusing. In contrast to the rod-shaped fiber optic waveguides typically found in immunofluorescent assay devices, in the present invention optical substrate **122** is of generally planar shape having two planar surfaces spaced by a width, as shown in FIG. **2**. Optical substrate **122** may for example be a square or rectangular glass microscope slide or coverslip, or the like. Materials for optical substrate **122** include glass, high-lead glass, quartz, optical plastic, and the like as are well-known in the art.

Light detection means indicated generally at **150** are positioned to detect fluorescent light emitted from biosensor **120**. The emitted light is reflective of the concentration of a selected analyte in a sample, as is better described subsequently in reference to FIGS. **2** and **7**–**10**. Light detection means **150** includes a collection lens **152** positioned to collect the emitted fluorescence from a plane parallel to and displaced from the surface of optical substrate **122**.

The distance **154** between collection lens **152** and optical substrate **122** is selected as known to those skilled to maximize the collection of light emitted from the region of evanescent light penetration. The light collected by collection lens **152** is then sent to detection means **150**, which responds by outputting signals reflective of the level of collected fluorescence light.

Detection means **150** may be any type of photodetector useful to detect light in the wavelength region spanning the wavelength range of the emitted fluorescence, as known in the art. However, in a preferred embodiment for simultaneous multi-analyte assays, detection means **150** is an imaging-type detector providing direct imaging of each of the fluorescent signal(s) originating in the evanescent zone **240**. In the apparatus of FIG. **1**, detection means **150** is a CCD (charge-coupled device) detector which produces a signal like that depicted in FIG. **4**C. Such imaging signal collection provides simultaneous measurement of multiple samples in a much simpler way than a system in which a separate optical element is needed to read each well or patch. The present imaging detection system also provides for collection of emitted fluorescence directly from the evanescent zone **240** (FIG. **2**), rather than via evanescent penetration and propagation of the fluorescence in the waveguide.

Alternatively, detection means **150** may be a photomultiplier, a semiconductor photodiode, or an array of

such detectors. In embodiments other than a CCD, an array is generally preferable to a single detector for some purposes. With an array of small detectors, the user can determine that the peak fluorescence is being detected and is not inadvertently missed due to misalignment of the collection and detection optics. Optionally, a grating spectrograph is coupled to the CCD or other detection means, to provide spectral analysis of the detected light. In that case, means are also provided to integrate the signal function around each peak to determine the total collected fluorescence from a sample. Alternatively, in an embodiment for use in a setting such as in a testing laboratory, and for which all the parameters of the assay have been standardized, the spectrograph may be replaced by a filter which passes only wavelengths in the region of tracer fluorescence.

FIGS. **14**A and **14**B depict an alternate and presently preferred embodiment of an imaging detection system, which may be substituted for the CCD imaging detector of FIG. **1**. In this embodiment, an array of photodiodes **680** is arranged with respect to a detection lens means such that light from a given patch is focused onto a corresponding photodiode (FIG. **14**A). In this embodiment, the detection lens means comprises a pair of opposingly oriented lenses **682**, **684**. The apparatus having the imaging detection system of FIGS. **14**A–B is substantially less expensive to make than one having a CCD detector. The patches **662**, **664**, **666** should be spaced appropriately to correspond to the size and spacing of the photodiodes, the focal length and magnification of the lens, and the distance between the waveguide surface, the detection lens or lenses, and the photodiode array, as generally understood in the art of optics.

Desirably, one or more filters **688** are positioned adjacent the detection lens, and preferably between two detection lenses as shown in FIG. **14**A. This arrangement provides for effective filtering of scattered excitation light and other stray light prior to impingement of the signal light on the photodiodes. The filter(s) can be of either bandpass or long-pass type, and the wavelength region to be passed will depend upon the wavelengths of the excitation light and the fluorescent light of the tracer molecule. For example, with laser diode excitation at 635 nm of the cyanine dye CY5 which has peak fluorescence at 670 nm, filters passing light longer than about 650 nm in wavelength are useful. In a presently preferred embodiment, a bandpass filter centered at 670 nm with a 40 nm line-width is used.

For focusing light beam **102** onto the end of the planar substrate waveguide, it is preferred to replace the typical spherical lens with a lens of semi-cylindrical shape, as better seen in FIGS. **3**C, **5**A, and **11**A. For purposes of this application, "semi-cylindrical" is defined to include both a transection of a right circular cylinder along a plane parallel to the vertical axis of the cylinder, and a transection of a right cylinder having bases of an elliptical shape. Thus, the lens shape may be similar to the type known as aspherical. A hyperboloid cross-section may also be suitable. The shape and dimensions of the curved surface of the lens should however be longitudinally uniform along the region used for focusing of the light beam.

As is better seen in FIG. **2**, optical substrate **124** is embodied as a planar waveguide having at least one planar surface **200** spaced from a second surface **201** by a width **202**. At least surface **200** is disposed in contact with a sample solution **203**. A plurality of capture molecules **204** are immobilized on surface **200**. The sample solution contains a plurality of analyte molecules **210** of a selected analyte, and a plurality of tracer molecules **220**. The capture molecules are chosen or constructed to bind to a binding moiety present on each of the analyte molecules **210**. The tracer molecule **220** is chosen or constructed to emit fluorescent light in response to stimulation by light of the appropriate wavelength. The level of fluorescence emitted by the tracer molecules **220** is a measure of the amount of analyte bound to the capture molecule and is thereby reflective of the concentration of analyte molecules **210** in the solution.

When light is being propagated in the waveguide **124** and internally reflected at the surfaces **200**, **201**, an evanescent light field is produced having an intensity curve **230** which drops off with distance from the surface **200**, as diagramed relative to a distance axis **232** and an intensity axis **234** (not to scale). An excitation zone **240** is the only region of the solution in which the evanescent light intensity is sufficient to excite a significant or detectable fraction of tracer molecules **220** (not to scale). Tracer molecules **220** outside zone **240** will contribute little or no induced fluorescence. Excitation zone **240** is typically between about 1000 and 2000 Å in depth.

Capture molecules **204** may be whole antibodies, antibody fragments such as Fab' fragments, whole antigenic molecules (haptens) or antigenic fragments, and oligopeptides which are antigenic and/or similar in 3-dimensional conformation to an antibody-binding epitope. Capture molecules **204** may also be a receptor molecule of the kind usually found on a cell or organelle membrane and which has specificity for a desired analyte, or a portion thereof carrying the analyte-specific-binding property of the receptor.

In FIG. **2**, a competition assay scheme is depicted (also termed a displacement assay). However, as will be apparent to the skilled person, alternate assay schemes such as sandwich assays may be performed with the present apparatus.

The capture molecules **204** may be immobilized on the surface **200** by any method known in the art. However, in the preferred embodiment the capture molecules are immobilized in a site-specific manner. As used in this application, the term "site-specific" means that specific sites on the capture molecules are involved in the coupling to the waveguide, rather than random sites as with typical prior art methods. Examples I–III detail methods for site-specific immobilization of capture molecules to the surface of the optical substrate by means of a protein-resistant coating on the substrate.

As previously stated, the intensity of evanescent light drops off rapidly with distance from the waveguide surface. Thus, only tracer molecules which are within an effective excitation range **240** (not necessarily to scale) from the waveguide surface, will be excited by the evanescent light to emit fluorescence. The range **240** is generally about 1000 to 2000 Å. This range is sufficient to ensure that essentially all tracer molecules **220** which are bound (directly or indirectly) to capture molecules **204**, will be detected, while the bulk of the tracer molecules which remain free in solution are outside the effective excitation range.

In a working embodiment of the apparatus of FIG. **1**, measurements of fluorescence are made by spectroscopy. For the examples involving rhodamine-tagged molecules, light source **100** is an argon ion laser (a LEXEL Model 95-2) at an emission wavelength of 514 nm. Fluorescence detection was done with a monochromator (SPEX Industries, Inc., Model 1680C) and a charge-coupled device (abbreviated CCD) (Photometrics Ltd. Series 200, or CH-250). Alternatively, light source **100** can be any light source emitting at the wavelength desired for excitation of

selected fluorescent dyes. Also, once an assay procedure has been validated and standardized, it may not be necessary to measure the fluorescence spectrum or spatial distribution of fluorescence. The detection means may be simplified in accordance with the minimum requirements of the assay.

In an alternate and presently preferred embodiment, light source **100** is a laser diode emitting in the red wavelength region of 600–700 nm, available from Toshiba (part no. TOLD 9211). This laser diode provides about 5 milliwatts of power with a peak emission wavelength of about 670 nm. Laser diodes emitting at 630 nm are also available and can be used. For an embodiment using wavelength in this region, it is necessary to use dyes such as cyanine dyes, whose fluorescence can be stimulated by excitation with wavelengths in the red spectral region. An example of such a dye is CY5, available from Biological Detection Systems, Inc., Pittsburgh Pa. (catalog no. A25000). The CY5 dye can be conjugated to the desired tracer molecule by the manufacturer's instructions and/or with a kit available from BDS. A second dye, CY7, which is available from the same source may also be suitable. The dyes and methods for conjugating are also characterized in the paper by Southwick, P. L., et al., titled "Cyanine Dye Labelling Reagents—Carboxymethylindo-cyanine Succinimidyl Esters", Cvtometrv 11:418–430 (1990). The use of laser diodes as a light source permits the biosensor and waveguide to be formed of plastic, thereby reducing the manufacturing expense and facilitating the integral molding of the semi-cylindrical lens with the waveguide and reservoirs.

In the embodiment of FIG. **2**, the immunoassay is a competition assay in which the tracer molecules **220** are constructed such that capture molecules **204** will bind analyte molecules **210** in place of tracer molecules **220**. Higher concentrations of analyte molecules **210** will cause most of the tracer molecules **220** to be displaced into the surrounding solution from capture molecules **204**, thus reducing the number of tracer molecules within excitation range **240** of the substrate **122**. This reduced binding of tracer molecules in turn reduces the amount of fluorescence. In contrast, lower concentrations of analyte molecules **210** will allow tracer molecules **220** to bind to capture molecules **204**, and thus to be held within the excitation range **240**.

In the embodiment of FIG. **1**, biosensor **120** is shown as a flow-through cell, shown in greater detail in FIGS. **3**A–B. A planar waveguide **302** which may be for example a microscope slide or coverslip, is sandwiched between two plates **304**, **306** which are held together by screw fittings **308**A, **308**B. A gasket **320** is seated between waveguide **302** and plate **306**. Gasket **320** is configured with two internal openings which, when gasket **320** is securely sandwiched between plate **306** and waveguide **302**, form reservoirs **322**, **324**. In reservoirs **322**, **324**, waveguide **302** constitutes one wall, plate **306** constitutes a second wall, and the inner edges **322**A, **324**A of the gasket form the remaining walls. Although the reservoirs **322**, **324** are here shown to be rectangular in shape, other shapes could be used. Also, instead of two reservoirs as depicted in FIG. **3**A, the gasket could have either just one opening or more than two, creating corresponding numbers of individual reservoirs.

Gasket **320** is preferably made of a semi-rigid material having an index of refraction less than that of the waveguide material in the wavelength range of the exciting light. For best results, it is believed that the index of refraction of the gasket material should be as low as possible compared to that of the waveguide. For a waveguide made of quartz or glass the index of refraction would typically be from about 1.46 to 1.52, higher for high-lead glass. A transparent (non-pigmented) silicon rubber (siloxane polymer) with an index of refraction of 1.35–1.43 is a presently preferred material for gasket **320**. TEFLON-type materials such as PTFE (polytetrafluoroethylene) or FEP (fluorinated ethylene propylene) have indices of refraction of around 1.34–1.35, and may also be suitable. However, because TEFLON surfaces tend to adsorb protein in a non-specific manner, silicon rubber is generally preferred.

The lower plate **306** in FIG. **3**B, has a pair of inlets **330**, **332** and a pair of outlets **340**, **342**. These inlets and outlets are arranged so as to permit solutions to flow separately through the respective reservoirs **322**, **324**. Desirably, the lower plate **306** may be made from aluminum alloy.

FIG. **3**C shows the waveguide **302** in isolation from the remaining parts of the biosensor. Lens **126** is shown receiving and focusing light beam **102** onto the waveguide. Desirably, the outer, surrounding edge **350** is coated with a reflective material, except for an uncoated region **352** at which the focused light from lens **126** enters the waveguide (FIG. **3**C). Arrows **354** indicate reflection from the coated edges. In FIG. **3**C, only one lens and one uncoated region are shown, however, for two or more channels, more portions of edge **350** may be left uncoated to allow light to enter the waveguide (see for example FIG. **4**A).

The reflective coating reflects back into the waveguide, light that would otherwise escape through the edge **350**. The intensity of the evanescent light wave is thereby enhanced. Suitable reflective coating materials include aluminum, silver, or the like, as known in the art. Alternatively, in place of a coating, reflectors could be positioned about the edges to reflect escaping light back into the waveguide.

The design with at least two individual reservoirs has significant advantages over a single reservoir embodiment for instances in which it is desirable to measure the test sample fluorescence simultaneously with fluorescence from a control region on the same waveguide. For example, the level of non-specific binding to the waveguide can be subtracted from the test sample fluorescence. Also, measurement changes due to fluctuations in intensity of the exciting light can be corrected for. In a displacement assay, the "control" region could be the pre-loaded waveguide with no analyte present in the sample, or with a known amount of analyte. With three or more wells, fluorescence can be measured for both a no-analyte control and at least one known, calibration analyte sample in addition to the "unknown" or test sample.

FIG. **4**A depicts the flow-through cell of FIGS. **3**A–**3**B as it would be used for a waveguide excitation protocol. Here, the light beam is split into two equal components **400**, **402** passing through respective focusing lenses **404**, **406** to illuminate "channel 1 " (CH.1) and "channel 2" (CH.2) in the waveguide **302**. Solid arrows **410** indicate the direction from which fluorescence in CH.1 is collected, while dashed arrows **412** indicate the direction from which fluorescence in CH.2 is collected.

When the focusing lenses **404**, **406** are properly aligned with respect to waveguide **302** and the light source, two illuminated strips **430**, **432** (FIG. **4**B) are visible which extend down the waveguide in the direction in which beam components **400**, **402** are aligned. Box **440** represents an approximate outline of the detection region of the CCD array while box **442** represents an approximate outline of the spectrograph entrance slit. As previously described, one embodiment of a detection system comprises a spectrograph in combination with a CCD array. FIG. **4**C depicts hypothetical expected results from such a detection system for

simultaneous measurement of a "blank" or no-analyte sample in CH.2 and a test sample or "unknown" in CH.1. Curves **450**, **452** respectively represent the fluorescence from CH.1 and CH.2. The fluorescent intensities of the blank and the sample would be compared by means of the values of the corresponding integrals of curves **450**, **452** over the respective regions **460**, **462**. A calibration curve for a series of calibration samples of known analyte concentrations would typically be made and used to determine the concentration of an unknown sample, as known in the art.

Of further interest in FIG. **4**A is the orientation of lenses **404**, **406** with respect to waveguide **302**. It will be seen that the curved edges **404**A, **406**A face towards waveguide **302**, which is 180° from the orientation depicted in FIG. **3**C. While the focusing lens can be oriented in either way, the arrangement of FIG. **4**A is presently preferred for illumination of the waveguide with the flow-through cell.

FIGS. **5**A–**5**D depict an alternate embodiment of a biosensor useful with the apparatus of FIG. **1**. The biosensor indicated generally at 500 has an integrally mounted or formed focusing lens **502** and waveguide **504** arranged such that lens **502** focuses light onto the forward end **506** of the waveguide. Focusing lens **502** is configured and positioned to focus a light beam onto the receiving end **506** of the waveguide **504** (FIGS. **5**A, **5**C). Side walls **511**, **512**, top and bottom walls **516**, **517**, and a removably sealing rear wall **518** enclose the space about the waveguide **504** to create reservoirs **520**, **522**.

The integral focusing lens **502** replaces focusing lens **126** in the apparatus of FIG. **1**. In the working embodiment of FIGS. **5**A–**5**D, the focusing lens is molded as part of the waveguide holder **500** of an optical plastic such as polystyrene, polycarbonate or the like.

Biosensor **500** also includes reservoirs **520**, **522** best seen in FIGS. **5**B, **5**C and **5**D in which sample solutions can be disposed. Optionally, for some applications it may be desirable to provide lengthwise ribs **530** (FIG. **5**D) along slot **504** which can define separate regions of the waveguide surface.

FIG. **6** depicts an alternate multiwell biosensor similar to that of FIGS. **5**A–**5**C, except that a series of discrete wells **600**, **602**, **604**, **606** are formed on the waveguide **504**. The embodiment of FIG. **6** would be used in a horizontal position, so that the wells **600**, **602**, **604**, **606** need not be covered.

The biosensor including the lens may be formed by molding of a suitable optical plastic. A holder comprising the reservoir walls, the lens, and frame elements as needed, may be pre-molded. A silica-surface waveguide is inserted subsequently with a refractive-index-matched adhesive to secure it in place and seal it as needed to create separate channels. Alternatively, the holder may be molded with a silica-surface waveguide in place, thereby eliminating the need for the adhesive.

In a presently preferred further embodiment, the waveguide is also formed of the optical plastic and is molded simultaneously with the lens and/or the reservoirs. The latter type construction is not suitable for use with excitation wavelengths of 488 to 515 nm, because known optical plastics tend to emit fluorescence when excited in this (the blue and green) wavelength region. This fluorescence would appear as background fluorescence. However, an alternate embodiment of the apparatus using a light source emitting at wavelengths of 600 nm and above, would accommodate a plastic waveguide. Molding the lens/wave-guide, or lens/waveguide/reservoir(s), as a single unit of plastic substantially reduces the cost of manufacturing and makes a disposable biosensor more feasible.

FIGS. **11**A–C shows another embodiment of a biosensor similar to that depicted in FIGS. **3**A–C. In FIG. **11**A, waveguide **10** is a glass coverslip inserted in a sawcut groove **12** (FIG. **11**B) in a solid, colorless plastic lens **14**. Transparent walls **16**, **18**, **20**, **22** are sealingly attached with index-matched adhesive to the lens **14** and about the edges of the waveguide **10** to form a pair of separate reservoirs **30**, **32** (FIG. **11**B).

In the embodiment of FIGS. **11**A–C, the curved forward edge **34** of the lens **14** is spaced at a distance **40** from the forward end of the waveguide **10**. Distance **40** is selected so as to match the focal length of the lens **14**. A mask **42** made of a material that is opaque to visible light, covers the rear edge **44** of lens **14**. The embodiment of FIGS. **11**A–C can be used in a vertical orientation as shown in FIG. **5**C. Alternatively, the biosensor may be oriented with the waveguide **10** in a substantially horizontal position, so that only one side of the waveguide **10** is used. In such case, a cap which can sealingly close the open ends of the reservoirs must also be provided. An advantage of the horizontal orientation scheme is that only a thin layer **50** of sample solution is required (FIG. **11**C). However, unless ribs along the waveguide **10** are provided, like ribs **530** in FIG. **5**D, the biosensor of FIG. **11**C in the horizontal orientation has only one effective sample channel.

While the curved edge **34** of lens **14** is shown as being substantially a semi right-cylinder in shape, other lens shapes are possible as described previously herein with respect to FIGS. **3**A and **5**C.

In a further and highly preferred embodiment of the biosensor depicted in FIGS. **12**A and **12**B, the end of the planar waveguide which is distal to the receiving edge, has a portion **650** of serrated or toothed shape. The angles 652A, 652B must be less than the critical angle for total internal reflection at the waveguide-air interface (the critical angle for polystyrene/air is about 51°). Preferably, the sum of angles 652A, 652B should be 90°, so that the light is retro-reflected back along the longitudinal axis of the waveguide; still more preferably, angle 652A=652B=45°. An advantage of this shape is that it increases the level of internal reflection without a reflective coating on the edges, thus reducing manufacturing complexity and costs. The increased TIR enhances the evanescent field intensity and thus improves sensitivity of the assay. Also, the serrated end edge helps to equalize (make more uniform across the whole waveguide) the intensity of light within the waveguide. The entire waveguide portion of the biosensor, should have an optical-quality surface, including the serrated end and the integral lens.

In a working embodiment, the waveguide is about 0.05 centimeters (cm) in thickness and the wells are about 0.08 to 0.1 cm in depth. The biosensor including the waveguide is about 2.5 cm wide and about 4.3 cm long.

The embodiment of FIGS. **12**A and **12**B also has a plurality of parallel wells **660** each extending along the longitudinal direction from the light receiving end **124** of the waveguide. Notably, each well contains a plurality of patches **662**, **664**, **666** each comprising a different immobilized Fab' species. The elimination of separation walls between such different species, which would extend crosswise to the direction of light propagation in the waveguide, further increases the sensitivity of the assay. The increased sensitivity results from 1) avoiding leakage of waveguide light through the walls, and 2) avoiding scattering of the excitation light which may excite unbound tracer molecules outside the region of evanescent penetration, undesirably increasing background fluorescence.

In another improvement, the sheet excitation beam is arranged to enter the receiving edge of the waveguide at an angle to the plane of the waveguide. FIG. **13** shows an angled integral lens **670** configured to accept such angled beam entry. For this purpose, the beam originating from the laser should be shaped to a sheet of width approximating the width of the receiving region of the waveguide, and of relatively narrow thickness (preferably no more than tenfold, and preferably one- to four-fold the waveguide thickness), using cylindrical and/or spherical lenses as known in the art.

The effect of so angling the beam entry is to increase the proportion of light exciting higher order modes that are propagated in the waveguide, thereby increasing the evanescent field intensity. The mean beam entry angle should be less than, but near the critical angle of the waveguide/solution interface. The closer the beam entry angle is to this critical angle, the greater the increase in evanescent intensity. However, the beam entry angle should be sufficiently below the critical angle to avoid the possibility of exceeding the critical angle due to imperfections in the optical manufacture or otherwise inadvertently increasing the amount of input light escaping at the waveguide/solution interface, which would increase the amount of fluorescence from free tracer (the background level).

FIG. **19** shows data for the fluorescence intensity of biotin-conjugated Cy5 dye bound to an avidin-coated silica waveguide (ch. A) as a function of beam entry angle, as compared to the "background" level of fluorescence from Cy5 dye free in solution (not conjugated to biotin and therefore not bound to the avidin-coated surface). The critical angle for TIR at the silica-aqueous solution boundary is about 26°. The data of FIG. **19** indicate that The signal-to-background ratio (ch.A÷ch.B) for a beam entry angle of 20° to 25° is about 4-fold higher than for 0°. Thus, beam entry angles of one to about five degrees less than the critical angle are presently preferred for silica waveguides. For a polystyrene waveguide with an aqueous adjacent medium, the critical angle for TIR is about 33°, and a useful range of beam entry angles is from about 25° to about 32°, with the higher angles generally being preferable.

The use of an angled beam entry necessitates adjustment of the orientation of the center of radius of curvature of the semi-cylindrical lens with respect to the waveguide receiving end, as will be evident to a skilled person. In a molded integral biosensor, the integral lens/waveguide may be formed as shown in FIG. **13**.

The following examples detail several methods for attaching the capture molecules to the waveguide surface in a site-specific manner. The general scheme for reducing the level of non-specific binding is to coat the waveguide with a protein-resistant material, and then immobilize the antibody to the coating. The scheme further includes derivatizing of the protein-resistant coating combined with site-specific modification of the antibody or other capture molecule to be immobilized, so as to provide site-specific attachment of the capture molecule to the coating.

Of the examples presented, the procedures of Examples I and II gave generally better results. At present, the avidin-biotin coupling method (Example II) is the most preferred. Using either coupling scheme, at least about 75% of the immobilized Fab' fragments were active, and the levels of non-specific binding were typically no more than 1–2% of the specific binding. The modified PEG coating gave slightly higher levels of non-specific binding, in the range of 5% to about 25%.

EXAMPLE I

PREPARATION OF WAVEGUIDE SURFACE—HYDROGEL

A silica surface was prepared with a hydrogel coating comprised of polymethacryloyl hydrazide (abbreviated PMahy). Fused silica slides of CO grade and thickness about 1 mm, available from ESCO, Inc., were suitable as waveguides (optical substrates).

To graft the PMahy to the silica, the surface was derivatized with aldehyde groups. The derivatization was accomplished by silanization with 3-aminopropyltriethoxy silane (abbreviated APS) to add an amino functional group, followed by reaction with glutaraldehyde to produce free aldehyde groups. The PMahy was then be reacted with these aldehyde groups to form the hydrogel coating.

Antibodies could be coupled to this hydrogel in at least two ways. In one method, the carbohydrate groups in the Fc antibody region are oxidized to aldehydes by treatment with sodium metaperiodate. However, few antigen-binding fragments contain carbohydrate moieties useful for this purpose. Thus, a preferred method comprised modifying the pendant hydrazido groups of the hydrogel to a maleimido group by treatment with succinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate (abbreviated SMCC; Pierce Chemicals). These maleimido groups can be reacted with the free thiol groups typically found in the C-terminal region of Fab' fragments, thereby coupling the Fab' fragments to the hydrogel.

Polymethacryloylchloride (abbreviated PMaCl) was prepared by radical polymerization of methacryloyl chloride (abbreviated MaCl) in dioxane under an inert atmosphere, as described in Jantas et al., *J. Polym. Sci., Part A: Polym. Chem.* 27:475–485 (1989).

A reaction mixture containing 21.1. mole % of MaCl, 78.1 mole % dioxane, and 0.8 mole % AIBN (azobisisobutyronitrile), was allowed to react for 24 hours at 60° C. with agitation. The PMaCl so produced remained in solution during the course of the reaction. The mixture was then diluted with twice the amount of dioxane used in the reaction and slowly added to an excess of hydrazine hydrate, to achieve a volumetric ratio of 2:5 for diluted PMaCl. The latter addition was carried out for about 30 minutes in an ice bath under a nitrogen atmosphere. The resulting mixture was then stirred for about an hour at room temperature. The product PMahy was purified by evaporation of dioxane and the remaining unreacted hydrazine hydrate, followed by washing in distilled water. The washed product was then dialyzed in a SpectraPor dialysis membrane having a molecular weight cut-off of 3,500 Dalton, to remove unreacted monomer.

The polymer so prepared was shown to have a molecular weight of about 26,000 as measured by gel permeation chromatography for the hydrochloride form. The concentration of polymer in solution in the hydrochloride form was estimated to vary between about 5% and 8% (w/v). It has been found that the polymer can be stored in aqueous solution at 4° C. under a nitrogen atmosphere, for at least 5 months without undergoing a detrimental amount of spontaneous cross-linking. Silica chips or glass or quartz microscope slides were cleaned with chromic acid, then treated with 5% APS/95% deionized water (v/v) for about fifteen minutes at room temperature. The APS-treated surfaces were rinsed with deionized water and absolute ethanol, and incubated in a vacuum oven which had been flushed at least three times with nitrogen, at 120° C. for 1 hour. The resulting silanized surfaces were then soaked in 2.5% glutaraldehyde (E.M. grade from Polysciences) in 0.1 M carbonate-bicarbonate buffer, pH 9.2, for two hours at room temperature.

Next, linear PMahy was reacted with the aldehyde groups on the treated chips to create a cross-linked polymer film with many unreacted hydrazido groups in the chains. This was done by dipping the treated chips in solutions of PMahy of between about 5% and 8% (w/v), pH 5.2, at a temperature between about room temperature and about 60° C., for a time sufficient to form a polymer film of thickness about 100 Å or less. The thickness of the hydrogel layer increases with time and temperature of incubation in the solution. It was found that optimal conditions for preparation of the film of 100 Å thickness or less, comprised incubating in 5% (w/v) PMahy for 2 hours at room temperature (about 25° C.).

Next, the free hydrazido groups of the polymer film were modified by treatment with SMCC to provide reactive maleimido groups on the ends of the polymer side chains. This was done by immersing the PMahy-coated substrates in a solution of 0.19% (w/v) SMCC in dimethylformamide for about 1 hour at 25° C.

Following derivatization with SMCC, the hydrogel-coated surfaces were treated with a 1 mg/ml solution of Fab' fragments in phosphate buffer, ph 6.0, with 5 mM EDTA. The waveguide surface so prepared was shown to immobilize Fab' molecules at a surface density of about 1.4×10^{-12} moles/cm^2. Also the surface was able to immobilize Fab' fragments at their C-terminal thiol groups in a site-specific way. The thickness of the resulting polymer film was determined by ellipsometry to be about 100 Å, as was desired. This film thickness is much less than typical previous polymeric films, which have thicknesses of 0.35 to 25 μm (microns). The above-described method of preparing the PMahy polymers is superior to that described by von Kern et al. using polymethacryloylacid esters. Such esters suitable for reaction with hydrazine hydrate often have a molecular weight of 80,000 Dalton or more, from which it is difficult to obtain a desirably thin film on the waveguide.

Finally, the Fab' fragments were coupled to the free maleimido groups pendant from the polymer-coated surface as follows. The prepared waveguide surface was incubated for 24 hours at 4° C. in a solution containing the Fab' fragments at a concentration of 1.5×10^{-7} molar, in a phosphate buffer with 5 mM EDTA (pH 6.0).

EXAMPLE II
PREPARATION OF WAVEGUIDE SURFACE—AVIDIN-BIOTIN

This strategy was designed to exploit the very strong binding affinity of biotin for avidin (binding constant of around 10^{-15}). An avidin coating was readily made by physical adsorption on a silica surface. The Fab' fragments were then conjugated with biotin to form biotin-Fab' conjugates, also referred to as biotinylated Fab' fragments or b-Fab' fragments. The biotin is coupled at specific location (s) on the Fab' fragments. The avidin coated surface is then treated with the b-Fab' fragments, so that the biotin binds to the avidin thereby immobilizing the Fab' fragment to the surface in a site-specific manner.

In actual experiments, the procedure was as follows. Chromic acid-cleaned silica surfaces were immersed in a solution of 3×10^{-6} M (molar) avidin for about 3 hours at room temperature. The surfaces were then washed several times in PBS to remove unabsorbed avidin.

Biotinylated Fab' conjugates were prepared from a solution of Fab' fragments in PBS (0.5–1 mg/ml), by addition of a sufficient amount of 4 mM biotin-HPDP in dimethylformamide to provide a 20-fold molar excess of biotin-HPDP. This mixture was incubated for 90 minutes at room temperature, and biotinylated Fab' fragments (abbreviated b-Fab') were purified by gel permeation chromatography with Sephadex G25 equilibrated in PBS.

An alternate method was used for biotinylating whole antibodies, in which biotin-LC-hydrazide was coupled to oxidized carbohydrate groups in the Fc region of the antibody. Mab designated 9-40 (a murine monoclonal IgG_1 antibody that binds fluorescein), was oxidized by incubation at a concentration of 1–2 gm/ml protein in 10 mM sodium periodate, 0.1M sodium acetate pH 5.5 for 20 minutes at about 0° C. Glycerol was then added to a final concentration of 15 mM to quench the reaction, and the mixture incubated a further 5 minutes at 0° C. The oxidized Mab 9-40 was purified by gel filtration chromatography on Sephadex G25 equilibrated with 0.1M sodium acetate buffer pH 5.5, and then reacted with 5 mM biotin-LC-hydrazide for 2 hours at room temperature with agitation. Unreacted biotin-LC-hydrazide was removed using a Sephadex G25 column equilibrated in PBS.

Avidin-coated surfaces were immersed in a 1.5×10^{-7} M solution of b-Fab'**0** fragments for about an hour at room temperature, followed by washing with PBS to remove unbound b-Fab' fragments. Optionally, polyethylene glycol (abbreviated PEG) was coupled to surfaces that were previously coated with the b-Fab' fragments, by immersion of the b-Fab'-coated surfaces in a solution of between about 5×10^{-8} and 1×10^{-7} M PEG. Unbound PEG was removed by washing in PBS.

The density of immobilized Fab' fragments obtained using the avidin-biotin coupling chemistry was about 1.4×10^{-12} moles per cm^2 (square centimeter).

EXAMPLE III
PREPARATION OF WAVEGUIDE SURFACE—PEG-TYPE

In this method, the terminal hydroxyl groups of polyethylene glycol (abbreviated PEG) were converted to primary amine or hydrazide groups by reaction with ethylenediamine (abbreviated ED) or hydrazine (abbreviated HZ), respectively, to produce PEG-ED_2 or PEG-HZ_2. The PEG molecules so modified were then coupled to APS-glutaraldehyde activated silica surfaces. One ED moiety on each PEG-ED_2 molecule couples to a free aldehyde group on the silanized-glutaraldehyde-treated waveguide surface. The other ED (or HZ, if PEG-HZ_2 is used) is then available to bind to an aldehyde moiety in a capture molecule (binding protein) such as an oxidized antibody or antibody fragment.

Monofunctional (PEG M2000, M5000) or difunctional (PEG 3400, PEG 8000, PEG 18,500) of the indicated molecular weights in Dalton, were reacted with p-nitrophenyl chloroformate (abbreviated p-NPC; obtained from Aldrich Chemicals) in solution in benzene. The mixture was agitated at room temperature for about 24 hours. Dry ethyl ether (less than 0.01% water, purchased from J. T. Baker Chemicals) was used to precipitate PEG-(o-NP)$_2$ from solution. The precipitate was vacuum-dried overnight. Between about 50% and about 100% of PEG molecules were converted by this treatment to PEG-Onp, as determined by hydrolysis with 0.1N sodium hydroxide to release the p-nitrophenol groups. The absorbance at 402 nm was determined spectrophotometrically and a molar extinction coefficient of 18400 M^{-1} cm^{-1} used to determine the amount of conversion. The level of conversion depended somewhat on the molecular weight of the PEG of MPEG.

PEG-(o-NP)$_2$ was then dissolved in ethylenediamine and agitated gently for about 3 hours at room temperature. The PEG-(ED)$_2$ was then precipitated by addition of a sufficient amount of dry ethyl ether. The yellow PEG-(ED)$_2$ solution was decolorized by addition of 1 drop of 12N (normal) hydrochloric acid, and the precipitation with ethyl ether repeated twice more. The wet PEG-(ED)$_2$ was dried under

vacuum overnight. Alternatively, in place of ethylenediamine, the PEG was derivatized with hydrazine to produce PEG-Hz_2.

The modified PEG was coupled to silanized-glutaraldehyde-treated waveguide surfaces prepared as described in Example I. A solution of 24 milligrams (mg) of PEG-ED powder dissolved in 1.2 milliliters (ml) of 0.15M PBS pH 7.4 or in the same volume of 11% potassium sulfate-sodium acetate buffer at pH 5.2. The prepared waveguide surfaces were immersed in the PEG-ED solution and incubated at 60° C. for about 24 hours. The procedure using K_2SO_4-acetate buffer yielded a higher density of PEG molecules attached to the surface than that using PBS buffer. Antibodies or other binding proteins were immobilized to the PEG-coated waveguides as follows. A solution of about 3 mg/ml of antibody was dissolved in 0.15M sodium acetate buffer, pH 5.2. A solution of equivalent weight of 50 mM sodium metaperiodate ($NaIO_4$) was then added, and the reactants were agitated at room temperature for about an hour. Unreacted sodium metaperiodate was removed by passing the reaction mixture through a desalting column (type PD-10 from Pharmacia), which had been pre-equilibrated with the sodium acetate buffer.

The PEG-coated waveguides were then incubated with the oxidized antibody solution in the sodium acetate buffer, pH 5.2, for 3 days at 4° C., then rinsed to remove unbound antibody.

Waveguides prepared by each of the coating procedures of Examples I–III, as well as by prior art random-site coupling methods, were analyzed to determine levels of non-specific binding relative to total fluorescence and amounts of immobilized antibody and of available binding sites. Comparative results of these analyses are shown in Table I.

The data in Table I were obtained using fluorescein-BSA (BSA=bovine serum albumen) conjugates with an epitope density of nine (that is, approximately nine fluorescein molecules bound per BSA molecule), and anti-fluorescein antibodies (designated Mab 9-40, or Fab' 9-40 for fragments). A hybridoma cell line secreting this antibody was obtained from Professor E. W. Voss of the University of Illinois at Urbana-Champaign. In these experiments and those whose results are shown in FIGS. **7**A, **7**B, **8**, **9**A–**9**F, and **10**A–**10**D, data acquisition and processing was accomplished using software supplied with the Photometrics Series 200.

Absolute antigen binding was determined by means of radiolabelled tracers or capture molecules. For example, radiolabelled BSA-FL_9, was allowed to be coupled with immobilized Fab' fragments for 5 or 60 minutes in phosphate buffer pH 7.3 at room temperature. The tracer concentration was 1.5×10^{-7} M. Three ml per sample of fluorescein-labeled BSA (BSA-FL_9) at concentrations ranging from 10^{-7} M to 10^{-7} M was injected into the flow cell. The injection was performed over a five-minute interval. The spectrum at wavelength of 488 nm was taken and the bulk BSA-FL_9 was removed by flushing with PBS buffer. Three more spectra were taken, and the fluorescein peak from 513 to 517 nm was integrated. These values were set versus the log of BSA-FL_9 concentration in order to obtain the binding isotherm.

For measurements of radioactivity, the coated silica chips with either immobilized radiolabelled capture molecules or with labeled tracer molecules bound to unlabelled capture molecules, were washed thoroughly in a suitable buffer and counted on a gamma counter. ^{125}I-labeled antibodies or antigens were preferred for the radiolabelling, which was done using the Chloramine-T method (see Greenwood et al., *Biochem. J.* 89:114–123 (1963)).

The levels of non-specific absorption of antigen on waveguides prepared by site-specific coupling with avidin-biotin (Example II; Table I rows 7 and 8 from the top) or hydrogel (Example I; Table I bottom two rows) were considerably better than most of the prior art coupling methods, being typically 1–3% (Table I). The results also indicated that non-specific binding to the avidin-coated waveguide was acceptably low for analyte molecule concentrations of less than about 10^{-5} M, without a wash step.

The percentage of immobilized molecules that were active (able to bind analyte) was also considerably higher for avidin-biotin and for hydrogel coupling chemistries, being in the range of 50% to 75% for Fabs. The results for IgG capture molecules coupled by heat treatment, acid treatment or by oxidation, indicated that only a low percentage of the IgG binding sites were active (Table I rows 1,2,4,5,6,10 from the top).

The row labeled silica-avidin with biotin-PEG, represents data obtained with the further refinement of preloading the surface (after attachment of the capture molecules) with biotin-PEG conjugates. This was done to passivate potential non-specific binding regions. However, the improvement obtained with biotin-PEG pre-loading was not large.

For the experiments whose results are shown in Tables II and III, Fab' fragments derived from a murine anti-human chorionic gonadotropin (anti-hCG) monoclonal IgG antibody were used. The parent monoclonal antibody was purified as described by van Erp et al. *J. Immunol. Methods*, 140:235–241 (1991). This mouse antibody, termed anti-hCG-A, is directed against a portion of the β-subunit of hCG (provided by Organon-Teknika of Boxtel, Netherlands). The whole monoclonal antibody anti-hCG-A was used in the experiments whose results are depicted in FIGS. **7**A, **7**B, **8**, **9**A–**9**F, and **10**A–**10**D. also indicated that non-specific binding to the avidin-coated waveguide was acceptably low for analyte molecule concentrations of less than about 10^{-5} M, without a wash step.

TABLE I

Summary of Solid-Phase Immunoassays using Silica Substrates

Surface[1]	Antibody[2]	Coupling Chemistry	Total Binding[3] ($\times10^{-12}$) (moles/cm^2)	Absolute Non-specific Binding[4] ($\times10^{-12}$) (moles/cm^2)	Relative Non-specific Binding (%)	Immobilized Antibody[5] ($\times10^{-12}$) (moles/cm^2)	Specific Activity[6] (%)
Hydrophobic Silica (DDS)	Heat Treated IgG	Random	0.65	0.04	6.46	3.00	21.67
Hydrophobic Silica (DDS)	Acid Treated IgG	Random	0.67	0.07	9.70	2.20	30.45
Hydrophobic Silica (DDS)	Fab' Fragment	Random	0.37	0.25	69.00	1.30	28.46

TABLE I-continued

Summary of Solid-Phase Immunoassays using Silica Substrates

Surface[1]	Antibody[2]	Coupling Chemistry	Total Binding[3] ($\times 10^{-12}$) (moles/cm^2)	Absolute Non-specific Binding[4] ($\times 10^{-12}$) (moles/cm^2)	Relative Non-specific Binding (%)	Immobilized Antibody[5] ($\times 10^{-12}$) (moles/cm^2)	Specific Activity[6] (%)
Silica/APS/GLU	Acid Treated IgG	Random	0.55	0.21	38.18	3.75	14.67
Silica/APS/GLU/PEG	Oxidized IgG	Specific	0.56	0.11	19.64	2.06	27.18
Silica/APS/GLU/PEG	Oxidized IgG	Random	0.41	0.10	24.39	1.44	28.47
Silica/Avidin	Biotin-IgG	Specific	0.72	0.02	2.92	0.94	76.60
Silica/Avidin	Biotin-Fab	Specific	0.84	0.02	2.62	1.10	76.36
Silica/Avidin (with biotin-PEG)	Biotin-Fab	Specific	0.80	0.02	1.88	"	72.73
Silica/Hydrogel (pre-swollen)	Oxidized IgG	Specific	0.17	0.01	6.88	7.95	2.14
Silica/Hydrogel (pre-swollen)	Fab' Fragment	Specific	1.51	0.03	2.03	2.76	54.71

[1]Abbreviations: DDS - dichlorodimethylsilane; APS - aminopropysilane; GLU - glutaraldehyde; PEG - polyethylene glycol (3400 MW); BSA - bovine serum albumin; IgG - intact immunoglobulin G; Fab' - antigen binding fragment with reactive thiol group; ND - not determined
[2]All immunoassays were performed with an IgG_1 monoclonal antibody (9-40) which binds fluorescein.
[3]Amount of ^{125}I-Fluorescein-BSA which bound to silica substrate.
[4]Amount of ^{125}I-BSA which bound to silica substrate.
[5]Amount of ^{125}I-9-40 immobilized on silica substrate.
[6]Percent of immobilized active sites which bound antigen molecules.

TABLE II

Summary of Solid Phase Immunoassays Using Silica Substrates Covered with Hydrogel with Maleimido Reactive Groups

Antibody	Binding Constant pK_0	Total hCG Immobilized Antibody ($\times 10^{-12}$ mol/cm^2)	Total hCG Binding in 5 min ($\times 10^{-12}$ mol/cm^2)	Total hCG Binding in 60 min ($\times 10^{-12}$ mol/cm^2)	Antibody Activity (%)	Absolute Non-specific Binding in 5 min (BSA) ($\times 10^{-12}$ mol/cm^2)	Relative Non-specific Binding in 5 min (BSA) (%)	Absolute Non-specific Binding in 60 min (BSA) ($\times 10^{-12}$ mol/cm^2)	Relative Non-specific Binding in 60 min (BSA) (%)
Fab' from Anti-hCG-A	8.85	1.39 ± 0.07	0.62 ± 0.03	0.81 ± 0.03	58.3 ± 0.8	<0.01	<0.97	<0.02	<2.47
Fab' from Anti-hCG-B	7.89	1.29 ± 0.06	0.51 ± 0.02	0.67 ± 0.03	51.9 ± 0.1	0.02 ± 0.01	3.85 ± 1.81	0.04 ± 0.01	5.91 ± 1.22
Fab' from Anti-hCG-C	8.70	0.74 ± 0.04	0.18 ± 0.01	0.41 ± 0.02	55.4 ± 0.3	<0.01	<6.22	0.03 ± 0.01	7.21 ± 2.09
Fab' from Anti-hCG-D	8.00	1.45 ± 0.06	0.58 ± 0.02	0.75 ± 0.03	51.4 ± 0.3	<0.01	<1.20	<0.02	<3.21
Fab' from Mouse IgG	—	2.50 ± 0.10	0.04 ± 0.01	0.06 ± 0.02	2.4 ± 0.7	—	—	—	—

The percentage of immobilized molecules that were active (able to bind analyte) was also considerably higher for avidin-biotin and for hydrogel coupling chemistries, being in the range of 50% to 75% for Fabs. The results for IgG capture molecules coupled by heat treatment, acid treatment or by oxidation, indicated that only a low percentage of the IgG binding sites were active (Table I rows 1,2,4,5,6,10 from the top).

The row labeled silica-avidin with biotin-PEG, represents data obtained with the further refinement of preloading the surface (after attachment of the capture molecules) with biotin-PEG conjugates. This was done to passivate potential non-specific binding regions. However, the improvement obtained with biotin-PEG pre-loading was not large.

For the experiments whose results are shown in Tables II and III, Fab' fragments derived from a murine anti-human chorionic gonadotropin (anti-hCG) monoclonal IgG antibody were used. The parent monoclonal antibody was purified as described by van Erp et al. *J. Immunol. Methods*, 140:235–241 (1991). This mouse antibody, termed anti-hCG-A, is directed against a portion of the βsubunit of hCG (provided by Organon-Teknika of Boxtel, Netherlands). The whole monoclonal antibody anti-hCG-A was used in the experiments whose results are depicted in FIGS. **7**A, **7**B, **8**, **9**A–**9**F, and **10**A–**10**D.

$F(ab')_2$ fragments were produced by digestion with pepsin using the procedure described by Grey and Kunkel, "H Chain subgroups of myeloma proteins and normal **7**S globulin," *J. Exp. Med.* 120:253–266, 1964. Following digestion, $F(ab')_2$ fragments were reduced to Fab' fragments using dithiothreitol (DTT). Specifically, 33 mg of purified antibody and 1 mg pepsin (Sigma) were dissolved in 0.1M sodium acetate buffer (pH 4.2) and the digestion was carried out at 37° C. for 16 hours. The digestion was terminated by adjusting the pH of the reaction mixture to 8.0 with 2 M TRIS base. The $F(ab')_2$ fraction was separated by gel permeation chromatography (Superdex Hiload, Pharmacia) using phosphate-buffered saline (PBS), pH 7.7, as eluent. Fab' fragments were prepared by reducing the $F(ab')_2$ fragments (1 mg/ml) with 1.75 mM DTT and 3.5 mM ethylenediamine tetraacetate (EDTA) in 0.17 M TRIS™ buffer (pH 7.4) for 45 minutes at room temperature. After

reduction, excess DTT was removed by gel permeation chromatography using a Sephadex G-25 column (Pharmacia) equilibrated in 0.1M sodium phosphate buffer (pH 6.0) containing 5 mM EDTA.

for this purpose for any known antigenic analyte molecule analyte can be obtained using the methods of Geysen et al. as disclosed in Patent Publication Nos. WO 86/86487 and U.S. Pat. No. 4,708,871, as well as in the scientific literature.

TABLE III

Summary of Solid Phase Immunoassay Using Silica Substrates with Adsorbed Avidin and Biotinylated Fab' Fragments

Antibody	Immobilized Antibody ($\times 10^{-12}$ mol/cm^2)	Total hCG Binding ($\times 10^{-12}$ mol/cm^2)	Specific Activity (%)	Absolute Non-specific Binding (BSA) ($\times 10^{-12}$ mol/cm^2)	Relative Non-specific Binding (BSA) (%)
Fab' from Anti-hCG-A	1.19 ± 0.02	1.22 ± 0.01	100 ± 5	0.05 ± 0.02	4.20 ± 0.02
Fab' from Anti-hCG-B	1.40 ± 0.05	1.38 ± 0.07	98 ± 9	0.05 ± 0.01	3.57 ± 0.02
Fab' from Anti-hCG-C	2.24 ± 0.02	1.10 ± 0.03	49 ± 3	0.05 ± 0.02	2.32 ± 0.01
Fab' from Anti-hCG-D	1.59 ± 0.02	1.24 ± 0.01	78 ± 2	0.05 ± 0.005	3.14 ± 0.01
Fab' from Mouse IgG	1.25 ± 0.02	0.03 ± 0.003	2.4 ± 0.05	0.09 ± 0.03	7.20 ± 0.03

In the experiments whose results are presented in Tables II and III, the specific binding values were determined using hCG labeled with ^{125}I as described for Table I, while ^{125}I-labeled BSA was used to measure the non-specific binding. In both Tables II and III, the immobilized antibody was anti-hCG A.

The fluoro-immunoassays of FIGS. **7**A, **7**B, **8**, **9**A–**9**F, and **10**A–**10**D and Tables I–III were performed using an interfacial fluorometer constructed at the University of Utah. Silica waveguides with the appropriate respective immobilized antigens were placed in the dual-channel flow-cell of FIGS. **3**A and **3**B. The two channels were used for sample and reference measurements, as described with respect to FIGS. **4**A–C. The light source was the 514.5 mn emission of an air-cooled argon-ion laser. The laser beam was split into two parallel beams, which were focused with lenses into the two channels of the waveguide. Fluorescence emission was recorded from 520 to 620 nm using a monochromator connected to a computer-controlled CCD camera. The fluorescence spectrum was integrated from 560 nm to 600 nm to improve the signal-to-noise ratio.

FIGS. **7**A, **7**B and **8** are charts depicting fluorescence intensity data obtained using two alternate formats for performing a fluorescence immunoassay to detect an antibody. In these experiments, the detection of antibodies to human chorionic gonadotropin (abbreviated hCG) was used as a model to determine which format provided the greatest sensitivity. It will be evident that the methods described could be adapted to the detection of any desired antibody in biological fluids such as plasma or serum, for example the detection of antibodies to proteins of viral and bacterial pathogens, depending only on obtaining the necessary antigen for use as the capture molecule.

For purposes of the tests shown in FIGS. **7**A, **7**B and **8**, the antibody to be detected (the analyte) was chosen to be a monoclonal antibody (designated anti-hCG-A) to an hCG antigen (the latter designated hCG-A). The data of FIG. **7**A were obtained with whole hCG molecules serving as the capture molecules (the antigen or analyte binding molecule) in the assay. The data of FIG. **7**B were obtained using an oligopeptide constructed to selectively bind the anti-hCG-A antibody, as the capture molecules. Oligopeptides suitable

To attach the necessary fluorescent dye, either the N-terminus of the oligopeptide was modified to provide an amino group for amino-reactive dyes, or the C-terminus was modified to provide a cysteine thiol group for thiol-reactive dyes). Preferably also, the complete oligopeptide sequence is of length sufficient that the attached dye is spaced from the binding site by at least two or three residues.

In the experiments of both FIGS. **7**A and **7**B, the tracer was a goat anti-mouse IgG labeled with tetramethylrhodamine (abbreviated TMR). For both assay formats, the capture molecule was biotinylated as described in Example II and immobilized on an avidin-coated silica substrate. The test antibody, anti-hCG A, was premixed with the tracer (goat anti-mouse IgG-TMR) in the test solution.

As will be understood by those in the art for a sandwich fluoroimmunoassay, the anti-hCG A antibody bound to the immobilized capture molecule, and the goat-anti-mouse IgG-TMR tracer in turn bound to the mouse anti-hCG A antibody. In this way a fluorescent sandwich formed on the substrate surface with the TMR-portion of the tracer molecule being held within the region of evanescent excitation.

The data of FIGS. **7**A and **7**B were obtained with the following protocol. Different concentrations of anti-hCG A were premixed with the tracer antibody (concentration fixed at 10^{-8} M) and injected into the sample channel. A 10^{-8} concentration of tracer antibody was also injected into the reference channel as a control. The fluorescence intensity of the sample channel was plotted vs. anti-hCG A concentration and the fluorescence intensity of the reference channel was also plotted on the same set of axes (this is really a plot of the non-specific binding of the tracer antibody vs. time, since no anti-hCG A was injected into the reference channel).

FIGS. **7**A and **7**B show the results for a sandwich assay format following the binding of anti-hCG A to immobilized hCG and to the oligopeptide, respectively. FIG. **8** shows the corresponding fluorescence enhancements for both cases. The data from FIGS. **7**A and **7**B were normalized for background fluorescence and re-plotted as fluorescence enhancement ($F_{sample}/F_{reference}$) versus log analyte concentration. The response curve was similar for both of the immobilized antigens (whole hCG and oligopeptide antigen)

over a range of antibody concentrations from 10^{-13} M to 10^{-10} M. However, whole hCG gave better precision.

It is also evident from FIGS. **7**A, **7**B and **8** that analyte levels (anti-hCG A) as low as 10^{-13} molar were detectable with the assay. In a further embodiment, the tracer antibody concentration is reduced to 10^{-10} M or less. This is expected to reduce background fluorescence due to non-specific adsorption of the tracer antibody and thereby further improve the sensitivity to 10^{-14} M or better.

FIGS. **9**A–**9**C depicts data obtained using an antibody as the capture molecule to detect an antigen, in a sandwich-type assay. As mentioned previously, two different antibodies are employed in a sandwich immunoassay—an immobilized capture antibody and a labeled tracer antibody in solution. Since the capture antibody and the tracer antibody must bind to distinct regions of the antigen, two different monoclonal antibodies which bind to different epitopes on the antigen are typically used in such assays. In addition to the anti-hCG-A, three other monoclonal anti-hCG antibodies (anti-hCG-B, anti-hCG-C and anti-hCG-D, respectively) were obtained from Organon Teknika which bound to different epitopes than did anti-hCG-A. Since only anti-hCG A is specific to hCG (the others also bind to certain hormones related to hCG), only six of the twelve possible pairwise combinations of antibodies provide strict selectivity for hCG.

FIGS. **9**A–F depict results obtained with different pairwise combinations, with Fab' fragments prepared from anti-hCG A (Fab'-A) and immobilized to waveguides using the avidin-biotin coupling chemistry. Fab' fragments prepared from anti-hCG B, anti-hCG C and anti-hCG D were labeled with tetramethylrhodamine for use as tracer antibodies (designated Fab'-B, Fab'-C and Fab'-D, respectively). FIGS. **9**A and **9**B show results with Fab'-B as the tracer molecule. FIGS. **9**C, **9**D show results obtained using Fab'-C as the tracer molecule. FIGS. **9**E, **9**F show results obtained using Fab'-D as the tracer molecule. Presently, Fab'-B and Fab'-C are preferred for use as tracers in an hCG assay.

An alternate format used a converse protocol, that is, Fab'-A as the tracer molecule and Fab'-B, -C or -D as the capture molecule. However, the format using Fab'-A as the capture antibody was generally superior in sensitivity. It can be seen from FIG. **9**B that hCG concentrations as low as 10^{-12} M could be detected by the assay with Fab'-A as capture molecule.

FIGS. **10**A–D show data obtained from a competition or displacement assay. Fab'-A fragments were immobilized to waveguides using either the avidin-biotin chemistry (FIGS. **10**A, **10**B) or the hydrogel coupling chemistry (FIGS. **10**C, **10**D). The immobilized Fab'-A fragments were pre-loaded with the tracer oligopeptide at a concentration of 10^{-8} M. Increasing concentrations of hCG were added to one channel of the flow cell (sample) and PBS buffer was added to the other (reference). For each coupling chemistry, the raw fluorescence intensities of the sample and reference channels are shown in the panels on the left (**10**A & **10**C) and the percent of full-scale fluorescence (in the absence of hCG) are shown in the panels on the right (**10**B & **10**D). The latter values were normalized for the change in reference fluorescence. Standard errors were plotted for all data points, but in some cases were smaller than the plot marks.

At present the sandwich immunoassay is preferred for several reasons. First, detection of concentrations down to at least 0.1 picomolar can be demonstrated, as compared to picomolar concentrations for the competitive assay. Also, the instant sandwich immunoassay was capable of detecting concentrations ranging over five logs—from 10^{-8} M to 10^{-13} M. Thus, a single assay formulation using the sandwich procedure could serve for a variety of applications where different detection limits are required.

A further embodiment of coating chemistry, and one which at present is highly preferred, provides for photo-activated coupling of the binding moiety (Fab fragment, antibody or whatever) to the waveguide surface. By combining the photo activation process with localized irradiation (for example, by masking), it is possible to sequentially couple different binding species to different regions of the waveguide surface. In this way, a waveguide surface patterned with patches each of a different capture molecule species (preferably Fab' fragments, though Fab fragments and whole antibodies or receptor molecules could be used) can be conveniently produced, without need for walls between the different species. For the present type of evanescent sensor, the elimination of unnecessary walls can significantly improve the sensitivity of the device by reducing background and enhancing evanescent field strength.

In a highly preferred embodiment, the coating chemistry also "passivates" the surface, that is, inhibits nonspecific binding of the fluorescent tracer, and thus reduces the background signal. As described in reference to Examples I–III previously herein, the presently preferred passivating strategy is to coat the waveguide with a very thin layer (preferably no more than about 3 to about 10 nanometers thick) of PEG (polyethylene glycol), having reactive side arms for attachment of the capture molecules.

A presently preferred coating is a type of compound referred to herein as a "block copolymer", comprising at least one hydrophilic block containing polymerized hydrophilic residues (polyethylene oxide, "PEO"), adjacent at least one hydrophobic block containing polymerized hydrophobic residues (polypropylene oxide, "PPO"). A subclass of such compounds referred to herein as "triblock copolymers" or "TBCPs", comprise a hydrophobic block flanked by hydrophilic blocks. A series of TBCPs are commercially available from BASF Corporation under the tradename PLURONICS. An example of a presently preferred compound is known generally in the literature as PLURONICS F108 or "PF108"; it has a molecular weight (MW) about 14,600 and the general formula $(PEO)_x(PPO)_y(PEO)_x$, where x=129 and y=56. In block copolymers, the hydrophobic PPO segment tends to adsorb strongly to plastics including polystyrene, leaving the PEO side-arms in a relatively mobile state. Block copolymers have the general property of inhibiting non-specific protein adsorption, while providing hydrophilic side chains useful to attach proteins, including Fabs or Fab fragments.

Also, while the present description is primarily with reference to PLURONICS-type compounds, it is within contemplation that other polymeric compounds having hydrophilic segments and hydrophobic segments and offering pendant OH groups for attachment of proteins or photo-activated linkers will be useful. As known in the art, these include SEPHAROSE-type materials and other polysaccharides. Also, block copolymers having polyurethane segments as the hydrophilic block may be useful.

Referring to FIG. **16**, a general procedure for preparing a patterned polystyrene waveguide is as follows. First, a waveguide surface **700** coated with PF108 molecules **702** is prepared. Next, the free PEO chain ends **704** of the PF108 molecules **702** in a selected region of the waveguide are derivatized in a photo-activated coupling reaction with a photoaffinity crosslinker **706**. Suitable crosslinkers are heterobifunctional reagents which have a photo-activatable group conjugated to a reactive functional group such as isothiocyanate, succinimide or maleimide. Upon irradiation

with light beam **701** of the appropriate wavelength (generally in the ultraviolet region), the photo-activatable groups of the crosslinker **706** react to covalently bind to the free PEO chain ends **704**. A mask **712** confines the irradiation to a first region **714** of the waveguide. The result is waveguide surface having reactive functional groups useful to bind Fab' fragments only in the first region **714**. Next, the waveguide surface is incubated with a solution of Fab' fragments of a first species (FAB 1 **720** in FIG. **16**) for a time sufficient to allow the binding of Fab' fragments to the derivatized region to go to completion. The unreacted Fab' fragments are then washed off, and the process of photo-activated derivatization is repeated for a second region of the waveguide, followed by incubation with a second species of Fab' fragment.

For coupling of Fab' fragments to a waveguide region derivatized with free maleimido groups (procedure of FIG. **16**), incubation with a solution of Fab' fragments can be performed substantially as described for the PMahy coating.

In an alternate embodiment, the Fab' fragments **720** are coupled to the crosslinker **706** before the crosslinker is photo-reacted with the PEO chain ends **704** (FIG. **17**). This embodiment is presently preferred because the surfaces thus prepared are capable of binding larger levels of analyte plus tracer per unit area than those prepared according to the protocol of FIG. **16**.

Suitable photoaffinity crosslinkers include aryl azides (amine-to-amine linkage), fluorinated aryl azides (C—H bond-to-amine linkage), and benzophenones (C—H bond-to-amine linkage or C—H bond-to-thiol-linkage, depending on the specific compound). Examples of each type are shown in FIGS. **18**A–**18**D, along with the corresponding photo-activated coupling reaction. Presently, benzophenones providing a C—H bond-to-thiol linkage are preferred, as these can be used to achieve site-specific coupling to Fab' fragments. Either the iodoacetamide or the maleimide derivatives of benzophenone ("BPIA" and "BPM", respectively) can achieve this purpose. At present BPM is preferred, as it exhibits a higher degree of specific binding and a lower degree of non-specific adsorption. This is because the coupling occurs via the C-terminal thiol groups of the Fab' fragments, as described previously herein for the PMahy coating. Other photo-affinity crosslinkers providing free maleimido groups may be equally suitable.

Table IV shows comparative data on the levels of specific binding and non-specific binding obtained for the crosslinkers BPM vs. BPIA, for surfaces which are uncoated or coated with one of four different TBCPs, and for the procedure of FIG. **16** vs. that of FIG. **17**. The TBCPs are PF108, and three others designated by the tradenames PP105, Pf68, and Pf88, also available from BASF. The respective PEO/PPO/PEO ratios and molecular weights of these compounds are 37/56/37 (PP105, MW=6500), 76/30/76 (Pf68, MW=8400), and 104/39/104 (Pf88, MW=11,400). As a model system, Fab' fragments derived from the 9-40 anti-fluorescein antibody were used as the capture molecules, with fluorescein-conjugated BSA representing the analyte. The BSA was radioactively labeled. Specific binding was determined as the binding of the fluorescein-BSA-conjugate, while nonspecific binding was determined from binding of native (unconjugated) BSA.

TABLE IV

Specific and Non-Specific Binding of Antigen to Antibodies Immobilized to Polystyrene Using Photo-Affinity Cross-Linking Reagents

Experiment No.	Pluronics Coating Time	Cross-linker	Fab' Concentration (Molar)	Irradiation Time (min.)	Specific Binding (SB) (mol. cm^{-2})	Non-Specific Binding (NSB) (mol. cm^{-2})	Relative NSB (NSB/SB) (Percent)
1. Fab	None	BPIA-Fab	5.00e-6	30	5.83e-13	6.74e-14	11.6
2. Fab	None	PS-BPM	5.00e-6	30	9.20e-13	3.71e-13	40.0
3. Fab	None	BPM-Fab	5.00e-6	30	1.00e-12	7.04e-14	7.0
4. PF108-Fab	24 hours	BPIA-Fab	5.00e-6	30	1.01e-12	1.95e-13	19.0
5. PF108-Fab	24 hours	PL-BPM	5.00e-6	30	1.74e-13	2.02e-14	11.6
6. PF108-Fab	24 hours	BPM-Fab	5.00e-6	30	1.35e-12	1.48e-13	11.0
7. PF108-Fab	24 hours	BPM-Fab	1.00e-6	30	3.79e-13	2.08e-14	5.5
8. PF108-Fab	24 hours	BPM-Fab	5.00e-7	30	3.40e-13	1.86e-14	5.5
9. PF108-Fab	24 hours	BPM-Fab	1.50e-6	20	2.92e-13	3.43e-15	1.2
10. PF108-Fab	24 hours	BPM-Fab	1.50e-6	10	3.86e-13	5.66e-15	1.5
11. PP105-Fab	24 hours	BMP-Fab	1.50e-6	10	3.66e-13	<1e-15	<0.3
12. PP105-Fab	24 hours	BPIA-Fab	1.50e-6	10	1.07e-12	3.61e-14	3.4
13. PP105-Fab	24 hours	BPIA-Fab	5.00e-6	30	6.78e-13	3.87e-15	0.6
14. Silica-MSil5000-Fab		BPIAFab	5.00e-6	30	1.17e-13	6.66e-15	5.7
15. Silica-MSil5000-Fab		BPIA Fab	1.50e-6	30	1.66e-13	Not Determined	—

In all experiments except Nos. 14 & 15, the substrate was a polystyrene surface. In expts. 1–3, there was no PLURONICS coating. In experiments 4–13, the surface was coated for 24 hours with a 4% w/v aqueous solution of the indicated PLURONICS compound. In experiments 1, 3, 4, and 6–15, the Fab' was coupled first to the crosslinker and the complex then photo-crosslinked to the substrate. In experiments 2 and 5, the crosslinker was first photo-coupled to the surface, then incubated with Fab. The concentration of crosslinker used was a 20-fold molar excess of the Fab' concentration.

In the photocoupling process, the amount of crosslinker coupled to the PF108 depends on the duration and intensity of the irradiation, the concentration of crosslinker molecules, etc. These variables can easily be tested and optimized to find parameters which will achieve a desired level of crosslinker and/or Fab' protein coupled to the waveguide surface. Generally, a Fab' concentration of about 0.5 mg/ml to about 1 mg/ml, and a 20-fold molar excess of crosslinker, are useful in the processes of FIGS. **16** and **17**.

Table V contains comparative data concerning the effect of different TBCP coating times on non-specific binding to PF108-coated polystyrene waveguides. As can be seen, the levels of non-specific binding achieved were indistinguishable for coating times at least as short as 10 minutes up to at least as long as 24 hours.

From the data in Tables IV and V, it is evident that the degree of non-specific binding was significantly lower for the PF108 and PP105 coatings than for Pf68 and Pf88, and with Fab' concentrations of 1.5×10^{6}M. For this reason, PF108 and PP105 are presently preferred BCPs. In general, among TBCP compounds, those exhibiting better resistance to non-specific binding (and thus presently preferred) are those having PPO segments of length about 45–50 residues or more. Desirably, the level of non-specific binding should be no more than about 10%, and preferably below about 1%–2%, of the level of specific binding. Alternately, or in addition, it is desirable that the absolute amount of non-specific binding be in the range below about 5×10^{-14} and preferably below about 5×10^{-15}. Neither the hydrophilic-to-lipophilic balance of the TBCP, the total MW, or the molecular weight ratio of PEO to PPO in the compound, appear to be as important as the length of the PPO segment in selecting TBCPs with good efficiency in inhibiting non-specific binding.

In addition to the TBCPs discussed above, diblock copolymers of PEO/PPO (DBCPs) will also be effective as waveguide coatings to inhibit non-specific binding. Here as with the TBCPs, those compounds having PPO segments of sufficient length, generally greater than about 40–45 residues, will be more effective. At present, TBCPs are preferred over DBCPs, because the PEO blocks are largely responsible for the non-specific-binding-inhibition properties of these compounds.

TABLE V

Effects of Pluronics Coating on the Non-Specific Binding of BSA and Fluorescein-BSA to Polystyrene

Experiment No.	Pluronics	Pluronics Coating Time	Fluorescein-BSA (mol. cm^{-2})	BSA (mol. cm^{-2})
1.	None	—	6.05e-12	8.37e-13
2.	F108	24 hours	5.46e-14	8.13e-15
3.	None	—	1.96 ± 0.04e-12	1.53 ± 0.06e-12
4.	P105	24 hours	<1e-15	<1e-15
5.	F68	24 hours	1.68 ± 0.36e-13	0.91 ± 0.32e-13
6.	F88	24 hours	1.03 ± 0.30e-13	0.32 ± 0.17e-13
7.	F108	10 min	<1e-15	<1e-15
8.	F108	30 min	<1e-15	<1e-15
9.	F108	60 min	<1e-15	<1e-15
10.	F108	180 min	<1e-15	<1e-15
11.	F108	24 hours	<1e-15	<1e-15

The above-described coupling scheme is very effective with a hydrophobic substrate such as a polystyrene waveguide, but less useful with silica-based substrates such as quartz, glass, and other silicon-based optical materials. Therefore, in an alternate embodiment of the photocoupling method for a waveguide made of a silicon-based material, a silica surface is treated with an undercoating to which a thin top coating of PEG polymer (the protein-resistant component) will effectively adhere. Three schemes for accomplishing this are described in detail herein; all use an undercoating which is a silica-affinic agent having a silyl group free to react with silica. The first scheme is described previously herein in Examples I and III. The second scheme uses avidin to couple a biotinylated-PEG to the surface. This scheme is similar in some respects to Example II, but is modified as described in Example IV. The third scheme is to use an undercoating which makes the silica surface hydrophobic (such as DDS, dichlorodimethylsilane, or DPS, diphenyldichlorosilane), and then to use one of the block copolymers as the top coating. Still another embodiment for use with silica surfaces employs a single coating of a silyl-modified PEG such as methoxy-poly(ethyleneglycol) trimethoxysilane ("PEG-silane") of molecular weight around 3500–5000.

The photo-linking process described above with reference to polystyrene surfaces can be adapted for any of these four silica coating schemes, preferably using a benzophenone photo-linker as described in reference to FIGS. **16** and **17** (Example IV). Similar considerations of the relative and/or absolute levels of non-specific binding apply in selecting preferred undercoating/top coating combinations. Example IV and Table VI describe experiments and results of several such combinations for use with silica-based substrates.

EXAMPLE IV

The specific and non-specific binding properties of four kinds of coated silica surfaces (silica-MSil(5000), silica APS-Glu-PEG(2000), silica-avidin-biotin-PEG(3400), and silica-DPS-PF108), to which Fab' fragments were photo-crosslinked with BPIA, were evaluated. MSil(5000) is the trimethoxysilane derivative of methoxy-PEG(5000), where PEG(5000) is polyethylene glycol of molecular weight approximately 5000. Diphenylsilane dichloride (DPS) is a generally hydrophobic compound which has an $SiCl_2$ group which can react with the Si—OH bonds in silica.

Silica-MSil(5000) surfaces were prepared by incubating silica chips for 40 min. at 90° C. with a 10% aqueous solution of MSil(5000). Silica-DPS-PF108 surfaces were prepared by immersing the silica chips for 1 h at room temperature in a solution of 10% DPS in toluene. The DPS surfaces were washed with ethanol, then with water, and then immersed in a 4% aqueous solution of PF108 for about 24 hours at room temperature.

Silica-APS-Glu-PEG(2000) surfaces were prepared by incubating silica chips with an aqueous solution of 10% APS at room temperature for 30 minutes. The APS-coated chips were then washed with ethanol and with water, and then immersed in 2.5% aq. solution of glutaraldehyde in bicarbonate buffer, pH 8.0, for 2 hours at room temperature. The chips were washed again, and then reacted with methoxy-PEG(2000) hydrazide in acetate buffer containing 11% K_2SO_4, pH 5.2 at 60° C. for about 24 hours. Silica-avidin-biotin-PEG(3400) surfaces were prepared by incubating silica chips with avidin (3×10^{-6}M in PBS) at room temperature for 3 hours, then washed with PBS. The avidin-coated chips were then reacted with biotin-PEG(3400) (also 3×10–6M in PBS) for 3 hours at room temperature.

TABLE VI

Expt. No./Coating	Specific and Non-Specific Binding of Antigen to Silica Surfaces: Concentration (Molar)	Irradiation Time (min.)	Specific Binding (SB) (mol. cm^{-2})	Non-Specific Binding (NSB) (mol. cm^{-2})	Relative NSB (NSB/SB) (Percent)
1. MSil [5000]	5.00e-6	30	1.2e-13	6.7e-15	5.7
2. MSil [5000]	6.00e-6	30	3.2e-43	2.97e-14	9.0
3. MSil [5000]	7.00e-6	45	3.0e-13	5.5e-14	18.0
4. MSil [5000]	7.00e-6	45	2.7e-13	3.3e-14	12.0
5. APS-Glu-PEG [2000]	6.00e-6	30	1.1e-13	3.3e-14	30.7
6. APS-Glu-PEG [2000]	6.00e-6	30	9.6e-13	1.9e-14	20.0
7. Av-Bio-PEG [3400]	6.00e-6	30	1.1e-13	3.2e-15	2.8
8. Av-Bio-PEG [3400]	6.00e-6	30	1.0e-13	3.2e-15	3.2
9. DPS-PF108	6.00e-6	30	4.Se-13	5.4e-14	12.0
10. DPS-PF108	6.00e-6	30	3.0e-13	5.7e-14	19.0
11. APS-Glu-PEG [2000]	6.00e-6	30	NA	3.0e-14	NA
12. APS-Glu-PEG [2000]	6.00e-6	30	NA	9.8e-15	NA
13. Au-Bio-PEG [3400]	6.00e-6	30	NA	2.9e-15	NA
14. Au-Bio-PEG [3400]	6.00e-6	30	NA	1.8e-15	NA
15. DPS-PF108	6.00e-6	30	NA	7.05e-14	NA
16. DPS-PF108	6.00e-6	30	NA	7.04e-14	NA

Silica chips were prepared as described in Example IV. In experiments 1–10, both specific and non-specific binding of antigen were measured for surfaces having a Fab' fragment of the 9-40 antibody immobilized by BPIA crosslinking to the indicated coating. The procedure of FIG. 17 (Fab' -BPIA conjugates prepared, then photo-linked to coating). In experiments 11–16, only non-specific binding to coated surfaces (no BPIA or Fab') was measured for each type of coating.

Fab'-photo-crosslinker conjugates were prepared by reacting Fab' fragments (of the 9-40 antibody) with benzophenone iodoacetamide (BPIA) at a molar ratio of 1(Fab'):20 (BPIA) in Hepes buffer, pH 7.4, at room temperature for 2 hours. The conjugated Fab' fragments were separated and purified by passing the reaction mixture through a PD-10 column in the same buffer, to remove the excess BPIA. The photo-linking reaction was performed by irradiating the prepared silica chips in a solution of Fab'-BPIA conjugates in a quartz cuvette with 295 nm light at room temperature for 30 minutes; the output of the light source was measured to be approximately 4.7 milliwatts per cm^2. Specific and non-specific binding were determined as for the data of Tables IV and V.

The results presented in Table VI indicate that of the above coating chemistries and using BPIA as the photo-linker, the avidin-biotin/PEG(3400) combination gave the best results, and the MSil(5000) gave the next best results.

It is also possible to use photo-crosslinking of capture molecules on a silica surface coated with APS only, by using an aryl azide (FIGS. **18**A–**18**D) as a bridge between the amino group in APS and the amine groups in the antibody (or other protein-type capture molecule). However, this protocol does not provide site-specific attachment of the antibody to the waveguide surface, since antibodies have plural amine groups, nor does it render the silica surface protein-resistant, and therefore is presently considered much less desirable.

The patterning processes described above require a suitable light source for localized crosslinker photo-activation. As described above, localized photo-coupling may be performed with an incoherent light source such as a xenon lamp with lens and a mask (FIG. **15**). A mercury vapor lamp with a 300 nm bandpass filter and a mask are another example of a useful incoherent light source. Alternatively, a coherent UV light source such as an argon ion laser may be used in combination with a translation stage. In the latter embodiment, a mask may or may not be needed to achieve the desired localized irradiation.

While many of the preceding experimental examples and results were obtained using hCG antigen/anti-hCG antibody and fluorescein/anti-fluorescein antibody systems, it will be understood by those skilled that the apparatus and the biosensor, as well as the site-specific waveguide-coupling methods and assay formats, all are applicable to assays for any antigen or antibody for which the requisite reagents such as appropriate capture molecules can be obtained, without undue experimentation. It will further be understood that while tetramethyl-rhodamine, fluorescein, and cyanine dyes are specifically mentioned as useful for labeling of tracer molecules, the apparatus and methods can also be useful with other fluorescent dyes capable of being conjugated to the desired tracer molecule.

Also, while the novel subject matter of this application is described herein primarily with respect to the apparatus in which excitation is by an evanescent field, the evanescent field is produced by directing a light beam into the edge or end of a waveguide, and the resulting fluorescence is directly collected from the evanescent zone (e.g., not via evanescent coupling back into the waveguide), the usefulness of many elements of both the optical and chemical portions of the subject matter is not so limited. Many elements in the instant subject matter will also be useful in alternate configurations of evanescent-light biosensors. One such alternate configuration is that in which the tracer molecules are excited by a non-evanescent light source, and the fluorescence is collected as evanescent light that propagates through the waveguide and is collected at the edge or end. Another such alternate configuration provides evanescent field excitation via a waveguide illuminated from the edge or end, with collection of fluorescent light by evanescent penetration back into the waveguide.

It will further be recognized that various modifications and substitutions may be made to the apparatus and the biosensor as described herein, without departing from the concept and scope of the invention.

What is claimed is:

1. An assay device comprising:

a housing having an exterior surface and sealing an interior area;

sample receptor means for receiving a sample containing an analyte selected for determining its presence, the sample receptor means being located on the housing's exterior surface;

sample treatment means for chemically reacting the sample with a self-contained reagent in a reaction zone to produce a reaction product mixture, the sample treatment means further transporting at least a portion of the reaction product mixture to a detection zone, the sample treatment means being sealed within a biosensor and in fluid communication with the sample receptor means;

detector means for responding to a physically detectable change in the detection zone which correlates with the amount of selected analyte in the sample and producing an electrical signal which correlates to the amount of the selected analyte in the sample, the detector means being oriented with respect to said biosensor and in optical communication with the sample treatment means; and

display means for visually displaying processed data collected from said detector means.

2. The assay device of claim **1** wherein the portion of the reaction mixture which is transported to the detection zone includes a reaction product with a physically detectable label which correlates with the amount of the selected analyte in the sample.

3. The assay device of claim **1** wherein the device further includes a power source in electrical connection with the detector means, the power source being sealed within the housing.

4. The assay device of claim **2** wherein the physically detectable label provides a change in reflectance or transmission of the detection zone, and the assay device includes a light source positioned to direct light on the detection zone and a light detector positioned to receive light reflected or transmitted by the detection zone to yield a respective reflectance or transmission output signal.

5. The assay device of claim **4** wherein said light source consists of a laser emitting diode in optical communication with the detection zone.

6. A method of determining the presence of one or more selected analytes in a sample, the method comprising the steps of:

introducing the sample to the sample receptor means of the assay device of claim **1**;

transporting the sample to the reaction zone within the sealed housing;

chemically reacting the sample with the self-contained reagent to produce a reaction product mixture in the detection zone;

producing a physically detectable change in the detection zone and an electrical signal which correlates with the amount of the corresponding selected analyte in the sample;

calibrating the physically detectable change using stored assay calibration information uniquely characteristic to the specific self-contained reagent and physically detectable change of the individual sealed housing; and

visually displaying the calibrated, physically detectable change.

7. The method of claim **6** wherein the method further includes the step of:

chemically reacting the portion of the reaction mixture transported to the detection zone with a second self-contained reagent to produce a reaction product with a physically detectable label which correlates with the amount of selected analyte in the sample.

8. The method of claim **6** wherein the method further includes the step of comparing the stored information with the electrical signal to determine the accuracy of the assay, the stored information including a pre-determined range for the electrical signal.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,316,274 B1
DATED : November 13, 2001
INVENTOR(S) : James N. Herron, Douglas A. Christensen, Hsu-Kun Wang, Karin Caldwell, Vera Janatová and Shao-Chie Huang

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [62], **Related U.S. Application Data**, change "continuation" to
-- continuation-in-part --

FOREIGN PATENT DOCUMENTS, change "(M)" to -- (MX) --

Item [56], **References Cited**, OTHER PUBLICATIONS, change "Portein" to
-- Protein --
Change "Science" to -- Sensors --
Change "Sit-e" to -- Site --
Change "*Biochemical*" to -- *Biomedical* --
Change "*Fluorscence*" to -- *Fluorescence* --
Change "Societym" to -- Society, --

Drawings,
FIG. 2, insert reference numeral -- 234 -- with its appropriate lead line
FIG. 3A, delete reference numeral "303" with its lead line
FIG. 4A, insert reference numerals -- 404A -- and -- 406A -- with their appropriate lead lines
FIG. 4B, insert reference numerals -- 440 -- and -- 442 -- with their appropriate lead lines
FIG. 5A, delete reference numeral "514" with its lead line
FIG. 19, in top right portion of figure, change "waveguide))" to -- waveguide) --

Column 4,
Line 42, after "less" change "that" to -- than --

Column 7,
Line 59, change "**124**" to -- **122** --

Column 8,
Line 8, change "**124**" to -- **122** --

Column 9,
Line 25, change "Cvtometrv" to -- *Cytometry* --

Column 11,
Line 32, change "waveguide holder" to -- biosensor --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,316,274 B1
DATED : November 13, 2001
INVENTOR(S) : James N. Herron, Douglas A. Christensen, Hsu-Kun Wang, Karin Caldwell, Vera Janatová and Shao-Chie Huang

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 16,
Line 6, change "gm/ml" to -- mg/ml --
Line 18, after "b-Fab"' delete "**0**"

Column 17,
Line 48, after "BSA-FL_9" delete ","
Line 54, change "10^{-7}" to --10^{-10} --

Column 19,
Line 67, change "βsubunit" to -- β-subunit --

Column 22,
Line 50, after "A 10^{-8}" insert -- M --

Column 26,
Line 15, change "Pf68" to -- PF68 -- and change "Pf88" to -- PF88 --
Line 18, change "Pf68" to -- PF68 -- and change "Pf88" to -- PF88 --
TABLE IV, in the 6th column entitled "Specific Binding" change "cm^{-2}" to -- cm^{-2} --
TABLE IV, 3rd column, 11th entry, change "BMP-Fab" to -- BPM-Fab --
TABLE IV, 3rd column, 14th entry, change "BPIAFab" to -- BPIA-Fab --
TABLE IV, 3rd column, 15th entry, change "BPIAFab" to -- BPIA-Fab --

Column 27,
Line 9, change "Pf68" to -- PF68 -- and change "Pf88" to -- PF88 --
Line 10, change "10^6M" to -- 10^{-6}M --

Column 28,
Line 67, change "10-6M" to -- 10^{-6}M --

Column 29,
TABLE VI, 4th column, 2nd entry, change "3.2e-43" to -- 3.2e-13 --
TABLE VI, 4th column, 9th entry, change "4.Se-13" to -- 4.5e-13 --

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,316,274 B1
DATED : November 13, 2001
INVENTOR(S) : James N. Herron, Douglas A. Christensen, Hsu-Kun Wang, Karin Caldwell, Vera Janatová and Shao-Chie Huang

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Please replace Fig. 2 with the following:

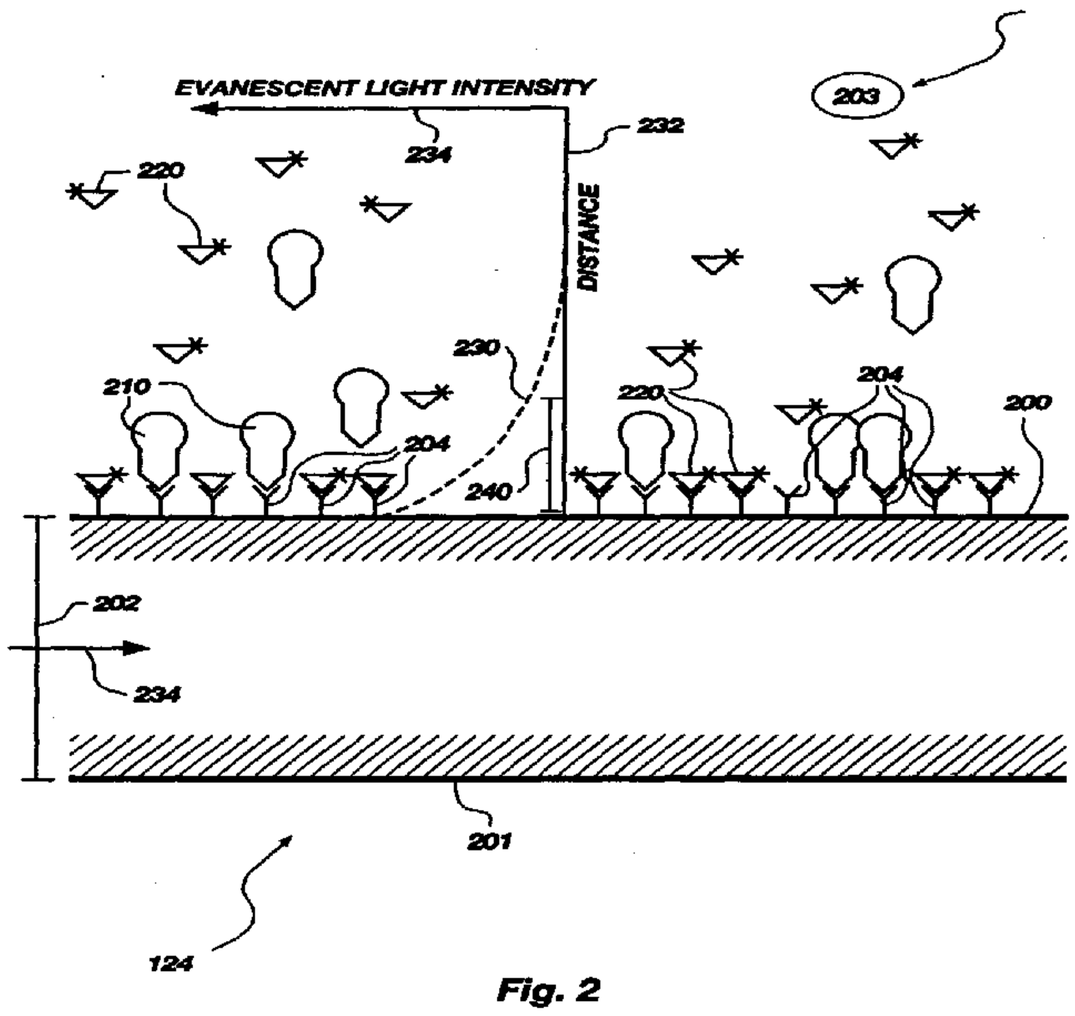


Fig. 2

Please replace Fig. 3A with the following:

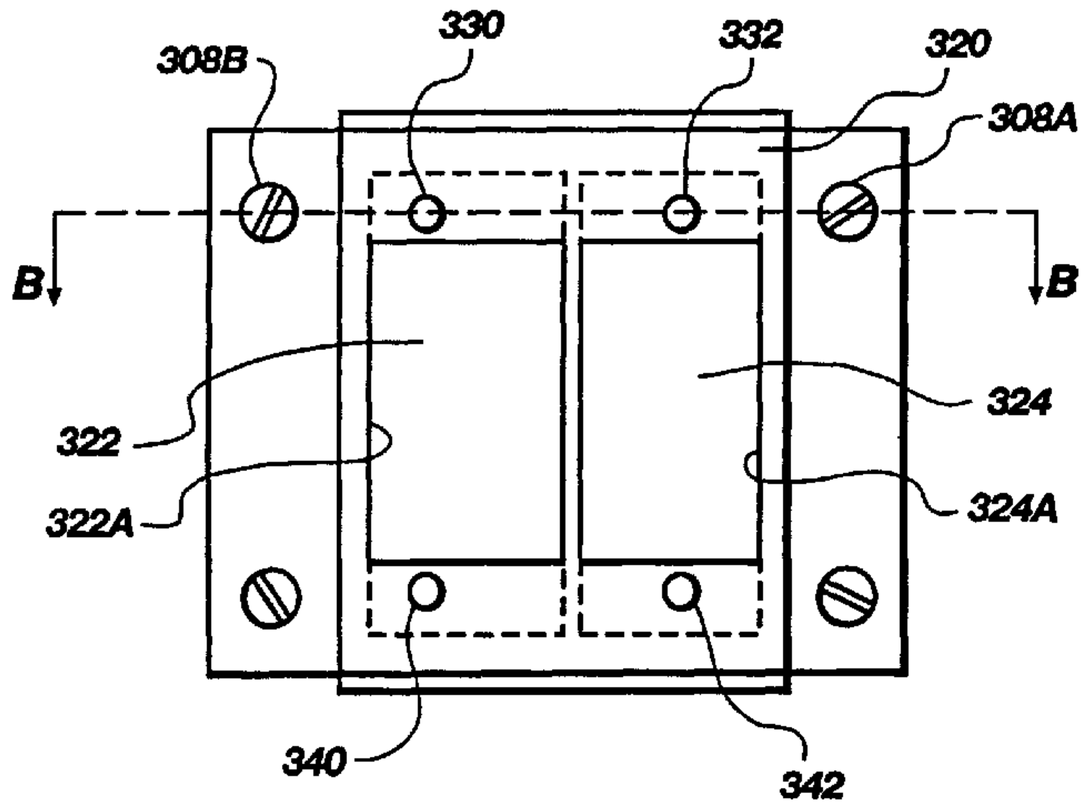


Fig. 3A

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,316,274 B1
DATED : November 13, 2001
INVENTOR(S) : James N. Herron, Douglas A. Christensen, Hsu-Kun Wang, Karin Caldwell, Vera Janatová and Shao-Chie Huang

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Please replace Figs. 4A and 4B with the following:

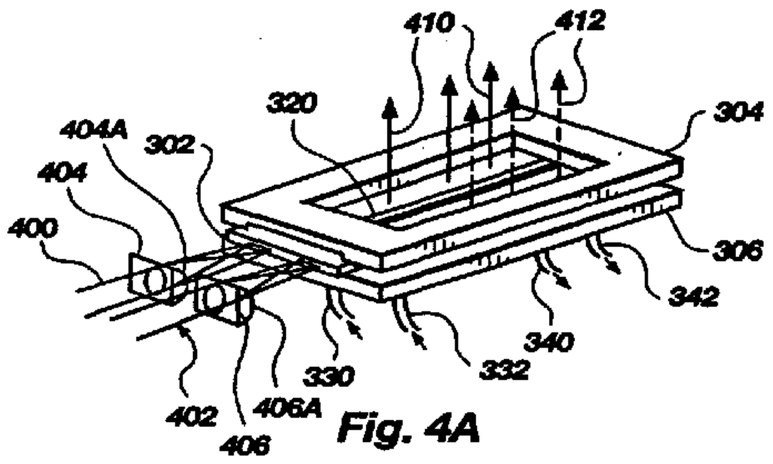


Fig. 4A

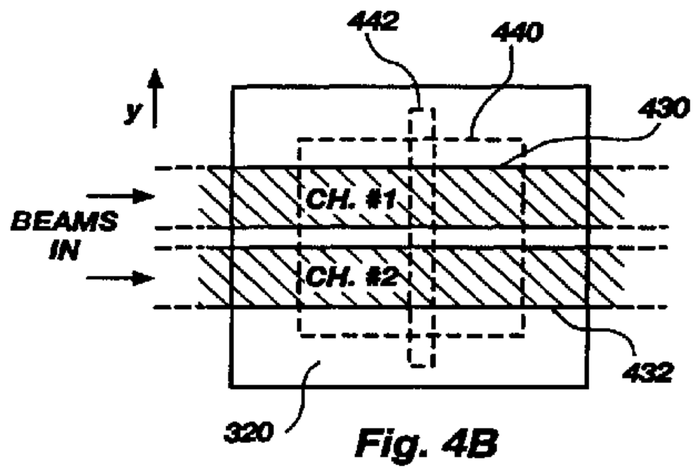


Fig. 4B

Please replace Fig. 5A with the following:

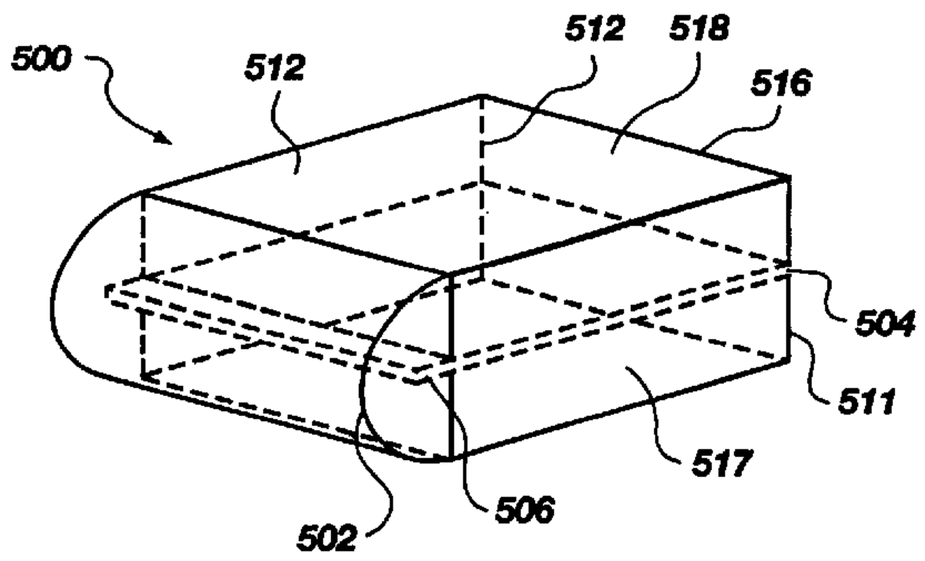


Fig. 5A

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,316,274 B1
DATED : November 13, 2001
INVENTOR(S) : James N. Herron, Douglas A. Christensen, Hsu-Kun Wang, Karin Caldwell, Vera Janatová and Shao-Chie Huang

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Please replace Fig. 19 with the following:

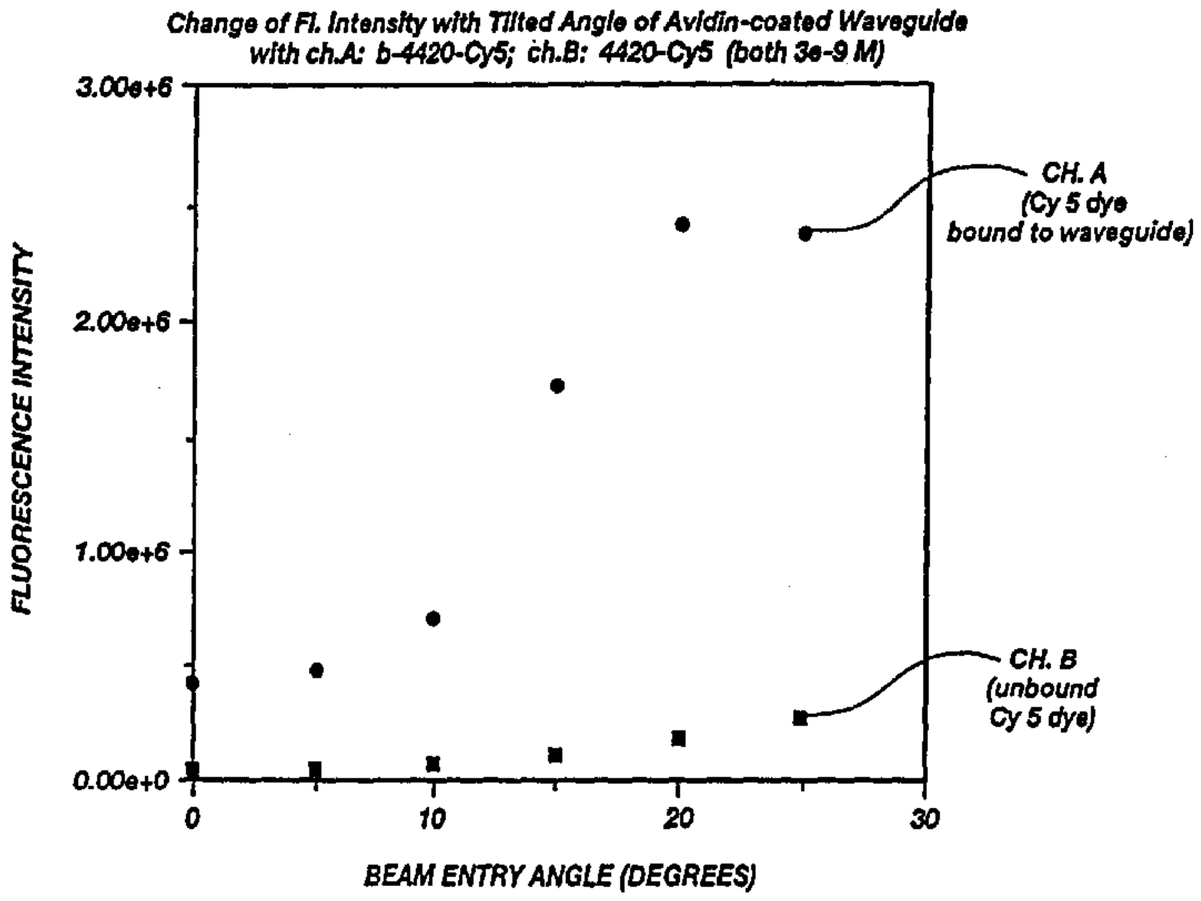


Fig. 19

Signed and Sealed this

Third Day of December, 2002

JAMES E. ROGAN
Director of the United States Patent and Trademark Office